ESTUDIO TÉCNICO Y BIOLÓGICO DE SISTEMAS AEROBIOS GRANULARES APLICADOS AL TRATAMIENTO DE AGUAS RESIDUALES HOSPITALARIAS

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Tesis doctoral, 2024

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TECHNICAL AND BIOLOGICAL STUDY OF AEROBIC GRANULAR SYSTEMS FOR HOSPITAL WASTEWATER TREATMENT

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Estudio técnico y biológico de sistemas aerobios granulares aplicados al tratamiento de aguas residuales hospitalarias

Memoria presentada por la MC.

Lizandra Pérez Bou

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Garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por el doctorando bajo la dirección de los directores de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han sutilizado sus resultados o publicaciones.

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No es, no, la luz del día
La que me llama,
Sino tus manecitas
En mi almohada.
Me hablan de que estás lejos:
¡Locuras me hablan!
Ellos tienen tu sombra;
¡Yo tengo tu alma!
Esas son cosas nuevas,
Mías y extrañas.
Yo sé que tus dos ojos
Allá en lejanas
Tierras relampaguean,
Y en las doradas
Olas de aire que baten
Mi frente pálida,
Pudiera con mi mano,
Cual si haz segara
De estrellas, segar haces
De tus miradas:
¡Tú flotas sobre todo,
Hijo del alma!

José Martí (fragmento del poema ¨HIJO DEL ALMA¨, 1882)

…para y por Ronaldo…

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LIST OF ACRONYMS

AD	Anaerobic digestion
AGS	Aerobic granular sludge
AMP	Ampicillin
ARB	Antibiotic-resistant bacteria
ARGs	Antibiotic-resistance genes
AZN	Azithromycin
BES	Bioelectrochemical systems
BOD	Biological oxygen demand
BOD_5	Biological oxygen demand on day 5
CAS	Conventional activated sludge
CCLP	Cyclophosphamide
CFD	Cefdinir
CIP	Ciprofloxacin
CLM	Clarithromycin
CMP	Carbamazepine
COD	Chemical oxygen demand
CTC	Chlortetracycline
DCL	Diclofenac
DOX	Doxycycline
ENR	Enrofloxacin
EPS	Extracellular polymeric substances
ERY	Erythromycin
ESBL	Extended-spectrum beta-lactamases
GEN	Gentamicin
HGT	Horizontal gene transfer
HRT	Hydraulic retention time
Kd	Sorption coefficient
KEGG	Kyoto Encyclopedia of Genes and Genomes
KTP	Ketoprofen
LC/TQ	Liquid Chromatography Quadrupole Time of Flight
LVX	Levofloxacin
MAT	Mean annual temperatures
MBR	Membrane bioreactor
MDR	Multidrug-resistant bacteria
MECs	Microbial electrolysis cells
MFCs	Microbial fuel cells
MGEs	Mobile genetic elements
MLSS	Mixed liquor suspended solids
NGS	Next generation sequencing
NOR	Norfloxacin
NPX	Naproxen
NSAIDs	Nonsteroidal anti-inflammatory drugs
OFL	Ofloxacin
OTC	Oxytetracycline
OTUs	Operational taxonomic units

LIST OF ACRONYMS (*cont.*)

PAO	Phosphate accumulating organisms
PCA	Principal component analysis
PCR	Polymerase chain reaction
PhACs	Pharmaceutical active compounds
PPCPs	Pharmaceutical and personal care products
qPCR	Quantitative polymerase chain reaction, Real-time PCR, quantitative PCR
RDA	Multivariate redundancy analysis
ROX	Roxithromycin
rRNA	Ribosomal RNA
SBRs	Sequencing batch reactors
SDZ	Sulfadiazine
SIMPER	Similarity percent analysis
SMX	Sulfamethoxazole
SMZ	Sulfamethizole
SPE	Solid phase extraction
SRT	Solid retention time
TC	Tetracycline
TMP	Trimethoprim
TRC	Triclosan
UHPLC	Ultra-High Performance Liquid Chromatography
WHO	World Health Organization
WW	Wastewater
WWTPs	Municipal wastewater treatment plants

SUMMARY

The discharges of low-quality hospital and urban wastewater (WW) effluents into receiving aquatic ecosystems cause ecological damage and human health problems by spreading high loads of nutrients, pathogens and toxic compounds. The current Urban Wastewater Treatment Directive (91/271/CEE, European Commission, 1991) regulates the limit values for the emission of organic matter, nitrogen and phosphorus from municipal wastewater treatment plants (WWTPs). Although conventional activated sludge (CAS) systems can often reduce the concentration of these pollutants to meet the required limits, these systems have several drawbacks, including high energy consumption, excess sludge production, significant greenhouse gas emissions, low quality of the treated effluents, and design efficiency challenges, especially when dealing with low strength wastewater or fluctuating influent rates. In addition, these systems are not specifically designed to target emerging contaminants such as pharmaceutical active compounds (PhACs), personal care products, and endocrine-disrupting chemicals. As a result, they often fail to effectively remove these contaminants. Despite the current lack of regulations setting concentration limits for antibiotics in WW treated effluents, the removal of these compounds in CAS-based WWTPs co-treating hospital and urban WW is often scarce, creating a significant hotspot for the spread and proliferation of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs), and contributing to the worldwide emergence of antibiotic resistance. Therefore, the search for alternative bioprocesses to overcome the drawbacks of CAS needs to be encouraged.

Aerobic granular sludge (AGS) operated in sequencing batch reactors (SBR) has a high potential for the improved treatment of urban and industrial WW and is currently considered the most promising technological alternative to overcome the disadvantages of CAS. Typical granular biomass allows the coexistence of different niches, enabling the metabolic versatility of the microbial communities and achieving excellent pollutant removal rates. The robustness of AGS for the treatment of complex WW containing recalcitrant and toxic compounds, such as antibiotics. Although several studies have investigated the feasibility of AGS for the efficient removal of PhACs in synthetic waters, to our knowledge, the evaluation of this technology for the treatment of real hospital WW has not been yet addressed. Thus, ***Chapter I*** focuses on the performance of AGS for the steady treatment of raw hospital effluents as a feasible approach to improve hospital WW

management technologies. The characteristics of the granular biomass were not significantly affected, and the main microbial communities (*Bacteria*, *Archaea* and Fungi) were progressively acclimated to the healthcare WW inlet over time. The removal of several common PhACs was additionally analysed according to their correlation with the study area or ecological risks. Good removal performances of antibiotics, antiepileptics, and antidepressants drugs were achieved.

In order to address the capacity of AGS to efficiently treat real hospital WW generating safe effluents, it is essential to determine the accumulation of ARGs and host ARB within the granular biomass. Therefore, ***Chapter II*** describes the development of an accurate methodology for the quantification of ARGs through sensitive and robust qPCR assays, supported by good primer design and validated protocols. The most relevant ARGs providing resistance to commonly prescribed antibiotic compounds were selected for their monitoring. The new molecular tools here designed were validated in samples retrieved from both natural and engineered environments, and the results highlighted a greater coverage of the new primer sets including a wider biodiversity of the bacterial populations carrying the different ARGs, plus a better design than the currently available molecular tools. Hence, after the validation of the new qPCR protocols, ***Chapter III*** provides an analysis of the occurrence and quantification of nine antibiotic compounds present in hospital WW and eleven ARGs within the AGS biomasses. Despite the excellent antibiotic removal performance achieved for the system, significantly high prevalence and enrichment of the targeted ARGs in the granular biomass were observed over time.

Finally, ***Chapter IV*** presents the profiles of ARGs of five WWTPs operated at low temperatures as a reservoir of psychrophilic ARB, highlighting the usefulness of the new qPCR assays to monitor the occurrence and fate of antibiotic resistance markers and evaluate the role of conventional bioprocesses as a hotspots of ARGs. This study is particularly important for several reasons: first, cold temperature environments are the most widespread extreme habitats of the Earth's biosphere with a resistome that remains largely unknown; second, the role of WWTPs in the cryosphere as hotspots for the dissemination of antibiotic resistance has not been well established yet, and finally, the contribution of urban WW influents to the abundance and prevalence of ARGs in activated sludge samples needs to be thoroughly explored under these extreme temperature conditions. The overall results suggest that ARGs are ubiquitous and highly

abundant in the cryosphere, despite the selection of microorganisms by low temperatures in WWTPs and natural cold environments. This study uses the recently developed quantitative methodologies for their application in environmental studies and strongly reinforces the global concern about the spread of antimicrobial resistance issues.

RESUMEN

La descarga de las aguas residuales hospitalarias y urbanas con altas concentraciones de nutrientes, agentes patógenos y compuestos tóxicos tiene graves efectos sobre los ecosistemas acuáticos receptores y en la salud humana. La actual Directiva sobre el tratamiento de aguas residuales urbanas (91/271/CEE, Comisión Europea, 1991) regula los valores límites para la emisión de materia orgánica, nitrógeno y fósforo en los efluentes generados en las Plantas de Tratamiento de Aguas Residuales Municipales (WWTPs). Sin embargo, los sistemas convencionales de lodos activados (CAS) implementados en las WWTPs a nivel mundial, presentan varias desventajas en su funcionamiento, entre las que destacan el alto consumo de energía, la producción excesiva de lodos, la emisión de gases de efecto invernadero y una baja eficiencia de eliminación, especialmente, cuando la carga contaminante en los caudales del afluente es alta y fluctuante. Además, estos sistemas presentan un diseño inadecuado que limita su rendimiento en el tratamiento de contaminantes emergentes, especialmente de compuestos farmacéuticos (PhACs), productos de cuidado personal y disruptores endocrinos. En particular, la alta prevalencia de antibióticos representa un desafío actual para el adecuado funcionamiento de los CAS, pues las concentraciones límites para su disposición final en los efluentes generados no están contemplados dentro del ámbito de aplicación de las normas regulatorias vigentes. Como consecuencia de este fenómeno, la contribución significativa de los CAS en la diseminación y proliferación de las bacterias resistentes a los antibióticos (ARB) y de genes de resistencia a los antibióticos (ARGs) ha sido ampliamente evidenciada, especialmente en las WWTPs que tratan efluentes urbanos y hospitalarios. Por lo tanto, ante la urgencia de superar estos inconvenientes, el desarrollo de bioprocesos prometedores y eficientes constituyen líneas de investigación priorizadas en una gran mayoría de países del mundo.

El sistema de lodos granulares aerobios (AGS) operado en reactores secuenciales discontinuos (SBR) es una biotecnología con un alto potencial para el tratamiento de aguas residuales urbanas e industriales. Actualmente, está considerada como la alternativa tecnológica más prometedora por sus múltiples ventajas con relación a los CAS. Uno de sus principales atractivos consiste en que las biomasas granulares permiten la coexistencia de diferentes nichos microbianos responsables de la versatilidad metabólica y de sus excelentes rendimientos en la eliminación de contaminantes. Además, se trata de

una tecnología robusta para el tratamiento de aguas residuales complejas con la presencia de compuestos recalcitrantes y tóxicos, por lo que se presentan como un bioproceso prometedor y económico para el tratamiento de aguas enriquecidas con compuestos farmacéuticos. Aunque varios estudios han determinado las capacidades de estos sistemas para la eliminación de PhACs en aguas sintéticas, según nuestro acceso a la información reciente, éste es el primer estudio que aplica esta tecnología para el tratamiento de efluentes reales de origen hospitalario. Por lo tanto, el ***Capítulo I*** evalúa el desempeño de un sistema aerobio granular en el tratamiento *in situ* de aguas hospitalarias crudas sin comprometer la estabilidad de operación del sistema, lo que permitirá mejorar la gestión de estos efluentes. Como criterios de selección de los PhACs se determinaron aquellos que se prescriben con mayor frecuencia, su correlación con el área de estudio y su potencial riesgo ecológico. De manera general, las propiedades de la biomasa granular no se afectaron significativamente y las principales comunidades microbianas (*Bacteria*, *Archaea* y Hongos) se aclimataron progresivamente de acuerdo con las características del influente a lo largo del tiempo de operación. En general, los efluentes hospitalarios mostraron un contenido de variable de PhACs, no obstante, se evidenció un buen rendimiento de eliminación de antibióticos, antiepilépticos y antidepresivos.

Por otro lado, es esencial determinar la acumulación de ARGs y de ARB en la biomasa granular para proponer finalmente un sistema AGS seguro y eficiente para el tratamiento de efluentes hospitalarios. Teniendo en cuenta este enfoque el ***Capítulo II*** describe el desarrollo de una nueva metodología para la cuantificación de ARGs mediante ensayos de qPCR precisos, sensibles y robustos, respaldados por un buen diseño de cebadores y protocolos de cuantificación adecuadamente validados. Se seleccionaron once ARGs que confieren resistencia a los antibióticos de uso más relevante, para su detección en muestras de ambientes naturales y diferentes bioprocesos de tratamiento de aguas residuales. El conjunto de nuevos cebadores contempló una mayor cobertura de la biodiversidad de estos genes y un mejor diseño con relación a las herramientas moleculares actualmente disponibles. Una vez validada la metodología cuantitativa, en el ***Capítulo III*** se describe su empleo para determinar el perfil de enriquecimiento de estos genes en la biomasa granular y evidenciar la relación de su abundancia con la de once compuestos antibióticos presentes en los efluentes hospitalarios. De esta manera, a pesar del buen rendimiento del sistema en la eliminación de los antibióticos, se evidenció una alta prevalencia y enriquecimiento de los ARGs en el periodo de evaluación.

Finalmente, el ***Capítulo IV*** se describen los perfiles de enriquecimiento de los ARGs seleccionados en cinco WWTPs que operan a bajas temperaturas como reservorios de ARB psicrófilas. La importancia de este estudio se fundamentó en que los ambientes templados son los hábitats extremos más extendidos de la biosfera, de los que se desconoce en gran medida su resistoma. Además, respaldó el uso potencial de los nuevos ensayos de qPCR para determinar la contribución de los bioprocesos convencionales como reservorios de ARGs y la influencia de los afluentes urbanos en la abundancia y prevalencia de estos genes. De manera general, los resultados sugirieron que los determinantes genéticos de resistencia a los antibióticos son ubicuos en la criosfera y en entornos naturales en regiones templadas, lo que refuerza fuertemente la preocupación mundial por la propagación de la resistencia a los antimicrobianos en múltiples ambientes naturales.

1-GENERAL INTRODUCTION

1

The material in the current introduction draws heavily on the author's previously published literature review:

Perez-Bou, L., Gonzalez-Martinez, A., Gonzalez-Lopez, J., Correa-Galeote, D., 2024. Promising bioprocesses for the efficient removal of antibiotics and antibiotic-resistance genes from urban and hospital wastewaters: Potentialities of aerobic granular systems. Environmental Pollution. 342, 123115.

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1.1 Global trends and prospects in the removal of emerging pollutants

Emerging contaminants are anthropogenic novel chemical compounds that occur at trace levels in air, soil, water, food, and living tissues and are currently unregulated (Rout et al., 2021). Among these contaminants, pharmaceutical active compounds (PhACs), such as antibiotics, antidepressants, analgesics, antiepileptics, antineoplastics, estrogens, radionuclides, metals, anaesthetics, lipid regulators, antipyretics, and solvents, are becoming increasingly common in various aquatic environments (Kahn et al., 2021) and are attracting the interest of the scientific community due to their toxic effects and inherently recalcitrant properties (Kristiansson et al., 2021).

There is considerable evidence that human activities have caused the accumulation, migration, and transformation of PhACs and their metabolites in the environment. In particular, the high concentrations of PhACs in wastewater (WW) from hospitals and health clinics (Azuma et al., 2016; Verlicchi 2017) eventually end up in wastewater treatment plants (WWTPs). Most of these compounds are more difficult to remove from WW than traditional pollutants (organic matter, phosphorus, and nitrogen), and their presence tends to disrupt the microbial communities of secondary treatment units in WWTPs (Verlicchi et al., 2015). As a result, WW has been identified as one of the major sources of PhACs and their metabolites in the receiving aquatic environments (Al Qarni et al., 2016).

Hospital WW contributes significantly to about 75% of the total PhACs load detected in the WW system (Santos et al., 2013; Oliveira et al., 2015). In particular, high proportions of anticancer drugs, close to 14% (Azuma et al., 2016), antimicrobials about 41%, and X-ray contrast agents ranging from 5-71% (Santos et al., 2013), have been reported. In this respect, the most prevalent PhACs in hospital effluents include cyclophosphamide (CCLP) and triclosan (TRC) (anticancer drugs), naproxen (NPX) and diclofenac (DCL (anti-inflammatory drugs), carbamazepine (CMP) (antidepressant drugs) (Gonzalez-Gil et al., 2016), and antibiotics from all therapeutic groups (Dinh et al., 2017; Wang et al., 2020; Nguyen et al., 2021).

Antibiotics are essential in treating bacterial infections in humans and animals (Puckowski et al., 2016) and have been hailed as one of the most important medical discoveries in recent history (Moser et al., 2019). However, global antibiotic consumption increased by approximately 35% in the early years of the 21st century (Knowles et al.,

2020; Schages et al., 2021). In this context, the overuse, misuse, or inappropriate use of antibiotics has led to their accumulation in several ecosystems, with a concomitant increase of up to 15-fold in antibiotic-resistant bacteria (ARB) in the environment (Couto et al., 2019; García et al., 2020) and in the abundance of antibiotic-resistance genes (ARGs) (Johnson, 2018; Perez-Bou et al., 2024b). The emergence and spread of ARBs have led to a reduction in the effectiveness of medical treatment against infectious diseases, resulting in higher morbidity and mortality, imposing huge costs on our society and representing a loss of the gains in terms of health since the discovery of antibiotics by Alexander Fleming in 1928 (Zaman et al., 2017). Hence, the antibiotic resistance phenomenon has led to the consideration of a global emergency for the World Health Organization (WHO) (WHO, 2014; Bondarczuk et al., 2016).

In general, the strategies for global environmental monitoring of PhACs and antibiotics must focus on identifying the most effective WW treatment technologies and determining the limits for their final disposal in receiving ecosystems (Azuma et al., 2018). Regarding the antimicrobial resistance issue, the policies should be able to rapidly identify hotspots for detecting clinically relevant ARB to prevent their spread, which is a fundamental challenge to ensure that antibiotics maintain their therapeutic utility. Another critical aspect is to determine the limits of the discharge of antibiotics, ARB, or ARGs into the sewage system to reduce the contribution to the evolution and spread of antibiotic resistance. Therefore, the identification and measurement of ARBs and ARGs in WWTPs must be improved to establish governmental policies to reduce the antimicrobial resistance phenomenon, as the quantification of the antibiotics, ARB and ARGs are critical aspects that must be monitored to maintain the efficacy of these essential therapeutic compounds for future generations (Pruden et al., 2021).

1.2 Antibiotics classification, modes of action and resistance mechanisms

Antibiotics are classified as J01 antibacterials for systemic use according to WHO anatomical and therapeutic chemical classification (WHO, 2017). There are several classes of antibiotics: beta-lactams, quinolones, tetracyclines, macrolides and sulphonamides, which vary in their molecular structure and physicochemical properties (Oberoi et al., 2019). The mode of action of antibiotics can be either bactericidal or bacteriostatic and are classified as broad-spectrum or narrow-spectrum based on their treatment selectivity. Depending on the type, antibiotic targets may include cell walls and

cytoplasmatic membranes, ribosomes, nucleic acids, bacterial metabolism reactions, and bacterial cellular enzymes (Figure 1). In these essential cell structures, antibiotic action results in inhibition of bacterial growth through various modes of action: inhibition of cell wall synthesis, disruption of cell membrane function, inhibition of protein synthesis by interactions with both 50s and 30s ribosomal units, inhibition of DNA and RNA syntheses, and action as antimetabolites blocking crucial steps of metabolic pathways (Bbosa et al., 2014; Oberoi et al., 2019).

Antibiotic resistance can be classified into natural (intrinsic: always expressed in the species or induced: only expressed after exposure to an antibiotic) and acquired resistance. On the other hand, acquired resistance consists of incorporating exogenous genetic elements (Impey et al., 2020). Although there are different mechanisms of acquired resistance (transformation, transposition, and conjugation), plasmid-mediated transformation is the most common route for acquiring exogenous genetic material (Reygaert, 2018). There are three basic mechanisms by which antibiotic resistance may occur in bacteria: reduction in the intracellular concentration of an antimicrobial, inactivation of an antibiotic, and loss of affinity of the antimicrobial target (Wright, 2010; 2011; Fernandes et al., 2013) (Figure 1). The most common mechanisms are the reduction of the permeability of the outer membrane and an increase in the natural activity of multidrug efflux pumps in mostly gram-negative bacteria (Cox and Wright, 2013), in addition to the lack of drug targets.

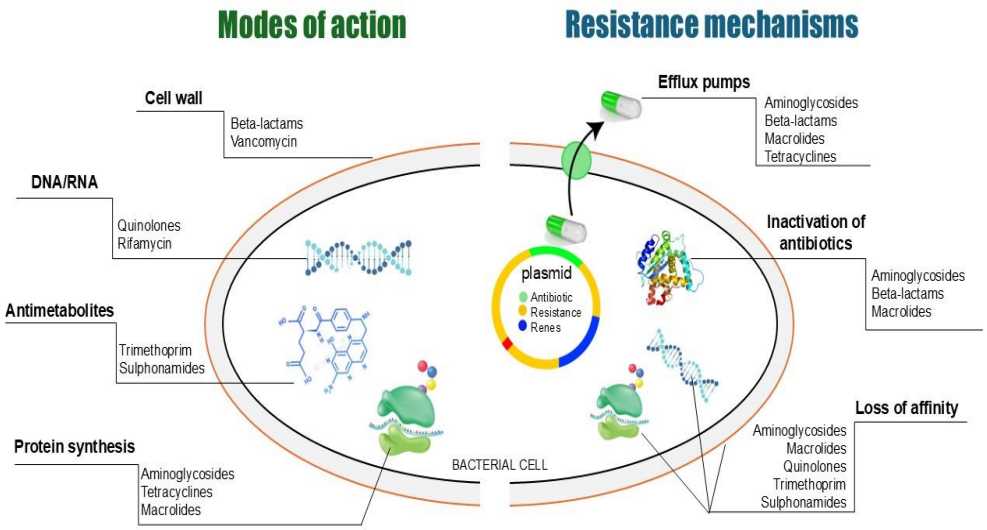


Figure 1. Scheme of the modes of action of common antibiotics to deal with bacteria and the most important mechanism by which bacteria become resistant. (Adapted from Wright, 2010).

1.3 Hospital wastewater as a hotspot of antibiotic resistance genes

Antibiotics, ARB, and ARGs discharged from hospital WW represent a serious biosafety risk with a substantial impact on the development of the antibiotic-resistance phenomenon through horizontal gene transfer (HGT) mechanisms in bacterial communities of WWTPs and natural ecosystems. It is worth noting that approximately 70% of the bacteria that produce nosocomial infections are resistant to at least one antibiotic (Chow et al., 2021). In addition, the slow development of novel antibiotics, notably for treating gram-negative bacterial infections, results in the growing use of last-resort antibiotics (colistin, carbapenems, or methicillin (Khan et al., 2020a; Nataraj and Mallappa, 2021)). This matter could be worsened if ARB transmits the determinant genetics of antibiotic resistance to non-pathogenic bacteria, which could serve as reservoirs of ARGs (Khan et al., 2021; Zhao et al., 2021a). Hence, multidrug-resistant and extensively drug-resistant bacteria involved in nosocomial infections constitute an alarming increment that must be closely monitored (Müller et al., 2018), as the overuse of last-resort antibiotics exacerbates the antibiotic-resistance issue (Marathe et al., 2017).

Many researchers have consistently reported the high concentration of antibiotics and potential ARG-host bacteria in hospitals and urban WW (Rodriguez-Mozaz et al., 2015; Aydin et al., 2019). Aydin et al. (2019) found total antibiotic contents greater than 300,000 ng L^{-1} in effluents from Turkish hospitals. Similarly, Rodriguez-Mozaz et al. (2015) described a maximum value of total antibiotic concentration of around 25,000 ng L^{-1} in Spanish hospital effluents. Generally, azithromycin (AZN), clarithromycin (CLM), norfloxacin (NOR), and trimethoprim (TMP) have been established as the most commonly found antibiotics in hospital WW (Omuferen et al., 2022). High levels of ARB are also found in municipal and hospital WW. In addition, the *Proteobacteria* and *Firmicutes* phyla are dominant in healthcare and urban settings, with differences in the abundance and composition at the class and genus levels (Selvarajan et al., 2021). An example of these differences is exposed in Figure 2. In hospital WW, the most abundant ARB genera include many common human pathogens such as *Acinetobacter*, *Aeromonas*, *Clostridium*, *Enterobacter*, *Enterococcus,* *Escherichia*, *Klebsiella*, *Pseudomonas*, *Ralstonia*, *Shigella*, *Spirochetes*, *Staphylococcus*, *Stenotrophomonas*, and *Vibrio*, among others (Santajit and Indrawattana, 2016; Dires et al., 2018; Buelow et al., 2020; Rahman et al., 2021).

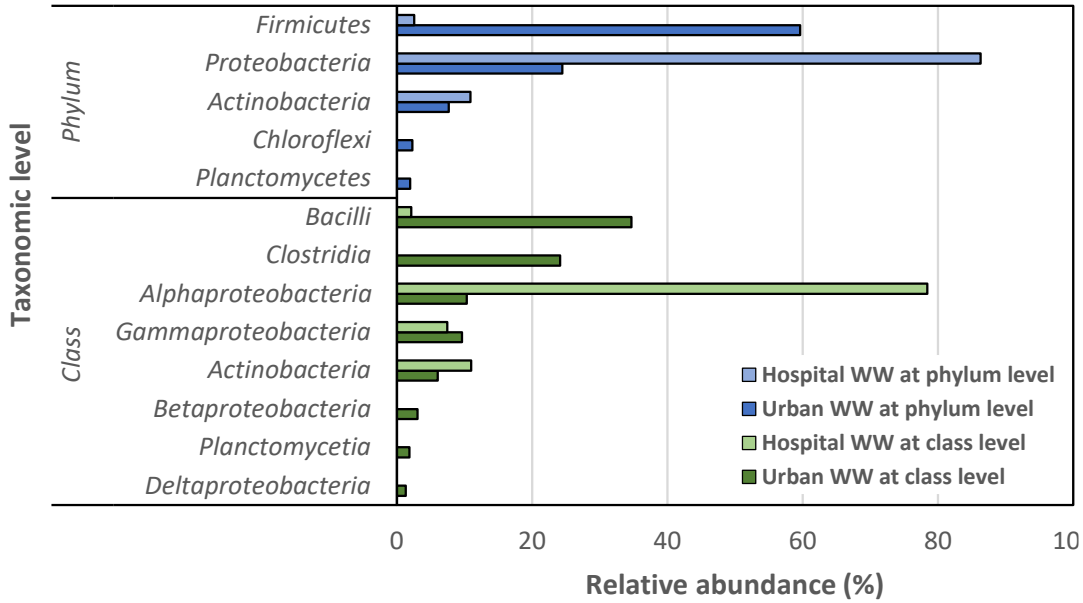


Figure 2. Taxonomic distribution profiles of bacterial communities in hospital and urban wastewater (WW). The data are from Selvarajan et al. (2021).

Several researches have also confirmed the prevalence of ARGs in hospital WW. Müller et al. (2018) showed that extended-spectrum beta-lactamases (ESBL) produced by gram-negative bacteria are omnipresent in hospital WW, as they were found in around 99% of different German hospital WWTPs. Consistently, Lorenzo et al. (2018) found increased abundances of sulfonamide (*sul*1) and beta-lactams (bla_{TEM}) resistance genes in Belgian hospital WW compared with influent and effluent of urban WWTPs. Paulus et al. (2019) found that hospital WW contained approximately 2-fold higher abundance of the *aph3a*, bla_{OXA}, bla_{SHV}, *sul*1, and *tetB* genes than those in urban WW in Delft and Nieuwegein (The Netherlands). Loudermilk et al. (2022) detected *Klebsiella pneumoniae* carbapenemase genes in hospital WW from Virginia (United States). Similarly, Proia et al. (2018) described a higher prevalence of clinically relevant carbapenemase genes (bla_{KPC}, bla_{NDM}, and bla_{OXA}) in Belgian hospital WW. In addition, recently, Zhu et al. (2022) and Li et al. (2022a) have found a broad prevalence of ARGs conferring resistance to the last-resort antibiotics, including the New Delhi metallo-beta-lactamase gene bla_{NDM}, mobile colistin resistance gene *mcr* and tigecycline resistance gene *tetX* in both influents and effluents from hospital WW treatment systems. Overall, health facilities are hotspots of the dissemination of antibiotic resistance, exhibiting a higher abundance of ARB and ARGs compared with other WW or aquatic environments.

Based on these considerations, hospital WW is an important hotspot in the enrichment of ARGs in both pathogenic and non-pathogenic bacteria due to the high concentration of antibiotic residues present in hospital WW effluents, including last-resort antibiotics (Zhang et al., 2020a; Li et al., 2022a; Zhu et al., 2022a). Therefore, proper treatments of the influents generated in hospitals and other healthcare services are mandatory to mitigate the emergence of antibiotic resistance, as this phenomenon could aggravate the medicinal ineffectiveness of antibiotics. In addition, addressing the appearance of novel antibiotic-resistance mechanisms, which first occur in healthcare services, is vital to prevent the spread of antibiotic resistance from hospital settings to other environments (Jindal et al., 2015).

For all these reasons, there is a growing interest in mitigating the presence of these micropollutants and their negative impacts on hospital WW, which has been largely neglected in current WWTP management standards (Figure 3).

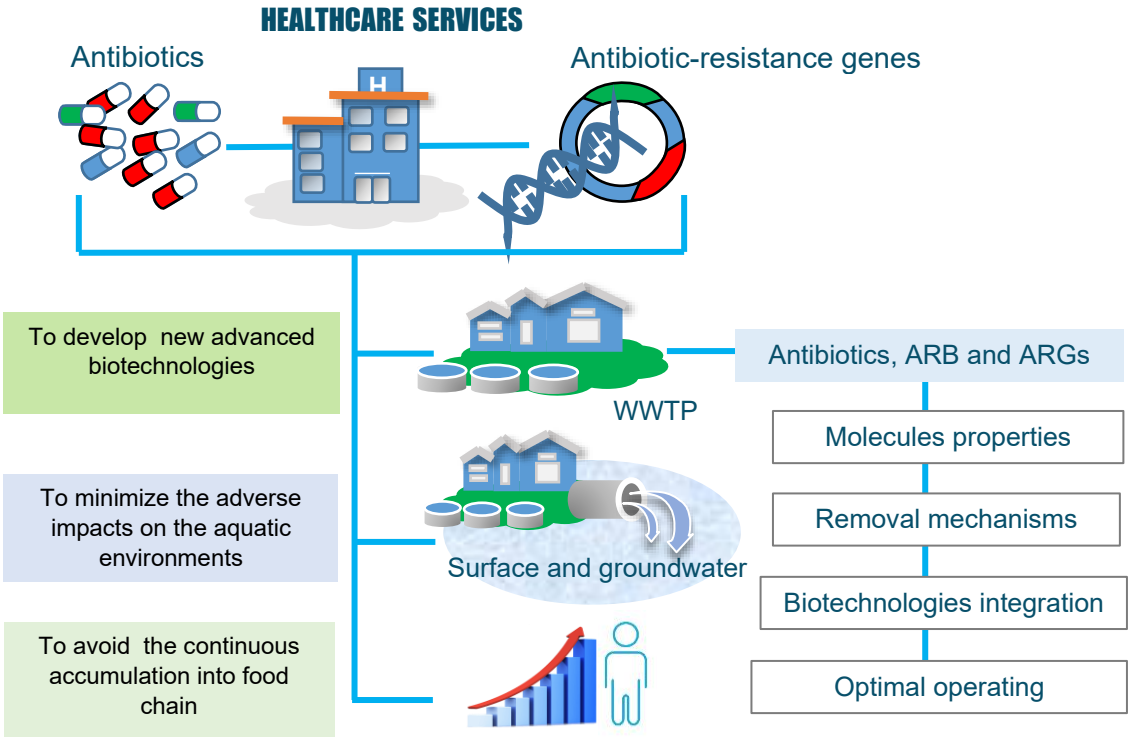


Figure 3. The main remarks regarding the research field of antibiotic and antibiotic-resistance genes (ARGs) removal from hospital wastewater to the environment. ARB, antibiotic-resistant bacteria; WWTP, wastewater treatment plant.

Given the importance of antibiotic resistance in hospital WW, monitoring antibiotic resistance in hospitals and the main representative bacteria that usually harbor ARGs in healthcare facilities are essential for ameliorate the harmful aspects of the antibiotic resistance issue under both the clinical and environmental aspects. Table 1 provides a comprehensive list of the ARGs most commonly found in WWTPs.

Table 1. The most commonly detected antibiotics worldwide and their associated antibiotic-resistance genes in wastewater treatment plants.

Antibiotic class	Antibiotic compounds	Antibiotic-resistant genes
Aminoglycosides	Kanamycin Tobramycin Gentamicin	*aadA*, *aacA4*, *aadB*, *aadE*, *strB*
Beta-lactams	Amoxicillin Cloxacillin Penicillin V Ampicillin	$bla_{\text{CTX-M}}$, bla_{TEM}, bla_{OXA}, bla_{SHV}, *mecA*
Macrolides	Clarithromycin Erythromycin Azithromycin Roxithromycin	*ereA*, *ermB*, *ermC*, *erm*43
Quinolones	Ofloxacin Ciprofloxacin Norfloxacin	*qnrS*, *qnrC*, *qnrD*
Sulfonamides	Sulfamethoxazole	*sul*1, *sul*2
Tetracyclines	Tetracycline	*tetA*, *tetB*, *tetE*, *tetG*, *tetH*, *tetS*, *tetT*, *tetX*
Trimethoprim	Trimethoprim	*dhfrA*1, *dhfr*14

1.3 Methods for surveillance of antibiotic-resistant bacteria

In general, two approaches have been used for antibiotic surveillance: culture-based methods and cultivation-independent techniques (nucleic acid-based methods) based on molecular metagenomics and polymerase chain reaction (PCR). Culture-based methods involve the isolation of target bacteria on general or selective culture media. Subsequently, the identification is determined based on the morphology, size, and pigmentation or by chemical profile using matrix-assisted laser desorption/ionization time of-flight mass spectrometry (MALDI-TOF) (Wieser et al., 2012). After isolation, the phenotypic antibiotic susceptibility is usually determined by disk diffusion or antibiotic gradient assays according to their growth response to specific antibiotic concentrations (Sukhum et al., 2019).

Traditionally, the bacteria addressed are clinical pathogens, such as *Acinetobacter*, *Campylobacter*, *Klebsiella*, *Pseudomonas*, and *Salmonella*, or common bacterial indicators of faecal contamination (*Escherichia coli* or total coliforms) (Davies and Davies, 2010; Sib et al., 2019). Besides, the bacteria that usually develop resistance to last-resort antibiotics-for example, gram-negative bacteria resistant to third-generation cephalosporins and carbapenems, particularly extended-spectrum beta-lactamase-producing *E. coli*, gram-positive vancomycin-resistant enterococci, and methicillin-resistant *Staphylococcus aureus*-are often included in routine culture-based determinations (Rahman et al., 2018; Schreiber et al., 2021).

The Monitoring and Evaluation Framework of the Global Action Plan on Antimicrobial Resistance of WHO recommends analyzing the prevalence of these ARB in high-, middle-, and low-income countries in both clinical and environmental samples (WHO, 2020). Despite the broad use of these methods in clinical settings, there are different drawbacks of this approach. One that stands out is the fact that some non-cultured bacteria can act as essential resistance reservoirs, which could result in lower detectability of the ARGs harbored by them. In addition, culture-based methods are time and labor-intensive.

The nucleic acid-based techniques rely on determining the whole antibiotic resistome of the samples by using next-generating sequencing or the specific quantification of a pool of ARGs using quantitative polymerase chain reaction (qPCR). Advances in the throughput of culture-independent methods have enabled to determine ARGs in isolates and environmental samples, meaning that they are now more readily available for both research and diagnostic microbiology (Haggard et al., 2016). Next-generation sequencing relies on collecting the biomass of interest, extracting DNA, preparing a library, and sequencing the whole genome. The antibiotic-resistant hosts can be identified from the assembly of the resistome with their phylogenetic biomarkers (16S ribosomal RNA (rRNA) gene) or the annotation of co-located genes (Nguyen et al., 2021). Although these methods have several advantages, their high cost and, the requirement of advanced facilities and highly specialized staff familiar with bioinformatics make their implementation difficult compared with other surveillance and monitoring methods. In addition, caution needs to be taken in interpreting the results due to the possibility of assembly errors inherent to this technique and for low-abundance ARGs, which may not be easily detected (Arango-Argoty et al., 2019).

On the other hand, qPCR enables the quantification of the total abundance of a specific ARG in an environmental sample (Bustin et al., 2009), making this technique the method of choice to specifically amplify ARGs quickly and easily, even when a gene is present at a low abundance. Hence, numerous qPCR assays have been reported to precisely quantify antibiotic-resistance genetic determinants and mobile element transfers in WWTPs (An et al., 2018; Müller et al., 2018; Buriánková et al., 2021; Keenum et al., 2022). Despite the advantages of qPCR, the presence of PCR inhibitors, long target amplicons, and the formation of nonspecific amplicons (e.g., primer dimers) can decrease the accuracy of the technique and must be evaluated correctly in each experiment (Bustin et al., 2009; Bustin and Huggett, 2017; Dreier et al., 2020; Ishii, 2020). It must be mentioned that the combination of different methods-parallel isolation of the bacteria, determining the resistome, and quantifying the specific genes-could resolve the particular issues of each technique (McLain et al., 2016). Finally, for routine monitoring of ARB or ARGs in WWTPs, the methods should provide quantitative information on removal efficiencies of ARB and ARGs during treatment processes and the potential human health risks of the effluents.

Several studies have focused on determining the abundance of ARGs in environments related to WW treatment. Marti et al. (2013) established the dissemination of antibiotics, ARB, and ARGs from WWTPs to riparian ecosystems. They compared the presence of a plethora of antibiotics, the prevalence of 11 ARGs, and the structure of bacterial communities between biofilms and sediment samples upstream and downstream of a WWTP discharging into the Ter River (Spain). They found relatively higher levels of CLM, SMX, TMP, metronidazole, and CIP (concentrations ranging from 0.0208 to 0.913 $\mu g\ L^{-1}$) after WW treatment. They also detected several ARGs, such as *qnrS*, *bla*$_{TEM}$, *bla*$_{CTX\text{-}M}$, *bla*$_{SHV}$, *ermB*, *sul*1, *sul*2, *tet*O, and *tet*W, in biofilms and sediments within the WWTP. The WWTP bacterial communities were dominated by common genera such as *Acinetobacter*, *Aeromonas*, *Exiguobacterium*, *Piscinibacter*, *Pseudohodoferax*, and *Pseudomonas*, all of them recognized pathogenic genera that may contribute to the occurrence, spread, and persistence of ARGs in the receiving aquatic ecosystems. Similarly, Szczepanowski et al. (2009) analyzed 140 different genes related to aminoglycoside, beta-lactam, chloramphenicol, fluoroquinolone, macrolide, rifampicin, sulfonamide, tetracycline, and TMP resistance mechanisms and multidrug efflux pumps in bacteria isolated from a German municipal WWTP. Out of these 140

genes, about 64 % were detected in final effluents, indicating that ARGs might be further disseminated to habitats downstream WWTPs. Moreover, they described that several genes had only been found in clinical isolates, demonstrating the occurrence of genetic exchange between clinical and WWTP bacterial communities.

1.4 Promising bioprocesses for the efficient removal of emerging pollutants

Conventional treatment of urban WW is not adequate for the removal of chemical and microbiological pollutants from hospital WW. Common procedures for WW treatment are based on physico-chemical and biological methods (Do et al., 2018). Physico-chemical alternatives such as photocatalytic oxidation, electrochemical oxidation and advanced oxidation are suitable for achieving high-quality effluents (Li et al., 2020a). However, in most cases, these biotechnological approaches are costly and not feasible to implement near hospitals prior to being discharged into the municipal sewer system (Nivedhita et al., 2022). On the other hand, biological processes stand out over physico-chemical ones because they require less energy and chemical input and mainly rely on transforming biodegradable organic contaminants into simple compounds via bacterial metabolism (Langbehn et al., 2021).

The biological processes are divided into the preliminary/primary, secondary, and tertiary stages, regardless of the biological technology employed (Nancharaiah and Kiran, 2018; Burch et al., 2019). Preliminary or primary treatments remove suspended solids and floating organic material for subsequent treatment. The elimination of antibiotics by this step is relatively poor (Verlicchi et al., 2012) due to the hydrophilic nature of these compounds (Caban and Stepnowski, 2021). The secondary treatment step aims to remove biodegradable organic matter and other nutrients via microbial oxidation of organic compounds (Verlicchi et al., 2012), and a plethora of metabolic mechanisms mediate the biotransformation of antibiotics as microorganisms utilize these compounds as carbon and energy sources (Oberoi et al., 2019). However, a high prevalence of the most crucial antibiotic groups and ARGs has been detected in WWTP effluents after secondary biological treatment (Marti et al., 2013b; Jurado et al., 2022). Finally, tertiary treatment relies on removing residual suspended solids and biological oxygen demand (BOD) and disinfecting effluents using UV radiation, ozonation, chlorination, or sand (Quach-Cu et al., 2018).

Conventional activated sludge (CAS), membrane bioreactor (MBR), and anaerobic digestion (AD) technologies have been explored extensively for WW treatment (Al Qarni et al., 2016; Caban and Stepnowski, 2021). Besides, innovative bioprocesses such as bioelectrochemical systems (BES) and aerobic granular sludge (AGS) are new alternatives to conventional methods (Cui et al., 2021; Hassan et al., 2021). Despite the advantages of the innovative technologies, some gaps must be filled to ensure the processes are efficient, simultaneously removing antibiotics and minimizing the abundance of ARB and ARGs in the final effluent. A representative summary of the current conventional and the new promising WW bioprocesses and their potential contribution to the efficient removal of antibiotics is presented in Table 2.

1.4.1 Conventional activated sludge

CAS systems are the standard techniques employed in the biological treatment of sewage, as it is the most common WW technology used worldwide (Verlicchi, 2018). The CAS procedure contributes to remove therapeutic PhACs classes (i.e., beta-blockers, nonsteroidal anti-inflammatory drugs, analgesics, and anticonvulsants) during secondary WW treatment (Al Qarni et al., 2016). However, CAS technology cannot wholly remove several classes of antibiotics, such as amoxicillin, CIP, clindamycin, OTC, SMX, and TMP (Bisognin et al., 2021; Li et al., 2022b). In addition, high concentrations of beta-lactams, fluoroquinolones, lincosamides, macrolides, metronidazole, sulfonamides, and TMP inhibit biological activities in WWTPs, reducing the quality of the generated effluents (Khan et al., 2021). Therefore, according to Langbehn et al. (2021), CAS systems cannot efficiently remove antibiotics in WW as this technology is not designed for their removal or metabolites and some derivatives. In addition, several studies have shown that the partial elimination of many antibiotics depends on their physicochemical properties and operating conditions, such as pH, nutrient supply, and redox conditions (Oberoi et al., 2019; Zhu et al., 2021). Rodriguez-Mozaz et al. (2015) revealed substantial concentrations of antibiotics and ARGs in hospital and urban influents, the generated effluents, and the receiving waterbodies (Ter River, Spain). Notably, fluoroquinolones were detected at the highest concentration, especially in the hospital effluent samples, and the levels of SMX and TMP were also high. In addition, although some antibiotics had reasonable removal rates, most antibiotics still presented in effluents after the WW treatment. Similarly, Aydin et al. (2019) detected high concentrations of CIP, CLM, and SMX in the effluents from 16 hospitals.

Table 2. Descriptive summary of different biological WW treatment systems for emerging pollutants removal.

Technology	Emerging pollutants	Removal (%)	Process	Remarks	References
CAS	**Antibiotics** Ofloxacin (OFL) Norfloxacin (NOR) Ciprofloxacin (CIP)	OFL: 21% NOR: 21% CIP: 43%	Glass flask lab scale/ synthetic WW	- Increases in the initial concentration of each antibiotic led to a decrease in the removal capacity due to saturation of active sites available for biosorption. - Negative charges on the active sludge surface provide more possible interactions with the positively charged antibiotics.	Ferreira et al. (2016)
	Antibiotics Azithromycin (AZN) Cefdinir (CFD) Ciprofloxacin (CIP) Clarithromycin (CLM) Levofloxacin (LVX) **Anticancer** Cyclophosphamide (CCLP) **Antiseptic** Triclosan (TRC) **Antidepressants** Carbamazepine (CMP)	AZN: 61% CFD: 95% CIP: 46% CLM: 60% LVX: 22% CCLP: 22% TRC: 22% CMP: 22%	Lab-scale batch reactor/ Real hospital WW and sewage	-The dominant removal mechanisms were found to be different for the individual antimicrobial compounds. -No significant differences in removal efficiencies were observed between both water samples, indicating the high reliability of the present lab-scale reactor.	Azuma et al. (2018)
	Antibiotics Ciprofloxacin (CIP) Clarithromycin (CLM) Sulfamethoxazole (SMX) **Anticancer** Cyclophosphamide (CCLP) **Antidepressants** Carbamazepine (CMP)	CIP: 99% CLM: 98% SMX: 97% CCLP:<10% CMP: >86 %	Real hospital WW	- High removal efficiencies of SMX and metabolites were observed under tropical climate conditions, and SMX was not found in the effluent. - Temperature was identified as the factor that influences removal efficiencies the most. - In addition to temperature effects, solar degradation could play a significant role in the removal of CMP. - The findings were unexpected; they suggest that conventional WWTPs could remove CMP under certain conditions, and tropical climate conditions are potentially more favourable than temperate ones.	Al Qarni et al. (2016)

Technology	Emerging pollutants	Removal (%)	Process	Remarks	References
MBR	**Antibiotics** Ciprofloxacin (CIP)	CIP: 91%	Anaerobic MBR/ Synthetic WW	-CIP significantly inhibited organic removal and methanogenic activity. -Microbial communities exposed to CIP did not differ significantly from the control in species diversity indices.	Do et al. (2018)
	Antibiotics Sulfamethoxazole (SMX) Trimethoprim (TMP) **Anticancer** Triclosan (TRC) **Anti-inflammatory** Diclofenac (DCL) Naproxen (NPX) **Antidepressants** Carbamazepine (CMP)	SMX: -43.9% TMP: 24.6% TRC: 100% DLC: -270.2% NPX: 23.6% CMP: -6.6%	Pilot-scale MBR/ Real hospital WW	- Adsorption and biodegradation varied depending on the compound properties. - SMX, TMP, CMP, NPX, and TRC removals were mainly done via an adsorption mechanism. -Microbial community capable of biodegrading PhACs was successfully developed in the MBR. - Only DCF and CMP were found biodegradable in batch experiments. - The majority of PhACs contained in hospital WW could be effectively removed in MBR and operated at a short HRT of 3 h. -Nevertheless, the significant accumulation of those compounds via adsorption onto colloidal particles in supernatant, which are subsequently separated by membrane filtration.	Prasertkulsak et al. (2016)
	Antibiotics Azithromycin (AZN) Ciprofloxacin (CIP) Erythromycin (ERY) Sulfamethoxazole (SMX) Trimethoprim (TMP) **Anticancer** Cyclophosphamide (CCLP) **Anti-inflammatory** Diclofenac (DCL) **Antidepressants** Carbamazepine (CMP)	AZN: 21% CIP: 51% ERY: <60% SMX: 7% TMP: 91% MTZ: 45% CCLP: <20% DCL: -5 % CMP: -6 %	Pilot-Scale MBR/ Real hospital WW	-The hospital WW does not significantly disturb biological processes in the MBR as a good performance was achieved. - No inhibition by antibacterial agents from the hospital was observed in the MBR. - The on-site biological WW treatment of hospital WW by MBR is insufficient to eliminate most of the target PhACs evaluated. - The hospital WW was found to be an adynamic system in which conjugates of pharmaceuticals deconjugate and biological transformation products are formed, which in some cases are PhACs.	Kovalova et al. (2012)

Technology	Emerging pollutants	Removal (%)	Process	Remarks	References
AD	**Antibiotics** Chlortetracycline (CTC) Oxytetracycline (OTC)	Acidogenic stage: CTC: 59.8% OTC: 41.3% Methanogenic stage CTC: 76.3%- OTC: 78.3%	Lab-scale Two-stage AD/Swine manure	- CTC and OTC negatively affected the bacterial community in methanogenic and acidogenic stages, respectively. - Archaeal diversity was reduced by CTC and OTC in the methanogenic stage.	Yin et al. (2018)
	Antibiotics Ciprofloxacin (CIP) Enrofloxacin (ENR) Sulfamethoxazole (SMX)	CIP: 92% ENR: 84% SMX: 100%	Full-Scale Biogas Plant/ Real WW	- AD process decreases concentrations of SMX, CIP, and ENR - A reduction of resistance genes was achieved by AD	Visca et al. (2021)
	Antibiotics Clarithromycin (CLM) Erythromycin (ERY) Sulfadiazine (SDZ) Sulfamethizole (SMZ) Sulfamethoxazole (SMX) Trimethoprim (TMP)	Thermophilic condition CLA: 36% ERY: 99% SDZ. 0% SMZ: 0% SMX: 98% TMP: 100% Psychrophilic condition CLA: 33% ERY: 20% SDZ. 0% SMZ: 0% SMX: 100% TMP: 100%	Bioreactor simulator/ Real fresh pig manure	-Some antibiotics (CLM, SDZ, and SMZ) were persistent in all experiments. -No adverse effects on biogas/methane yield during digestion were observed in the batch tests under antibiotics.	Feng et al. (2017)

Technology	Emerging pollutants	Removal (%)	Process	Remarks	References
AD	**Antibiotics** Sulfamethoxazole (SMX) Trimethoprim (TMP) Erythromycin (ERY) Roxithromycin (ROX) **Antiseptic** Triclosan (TRC) **Anti-inflammatory** Diclofenac (DCL) Naproxen (NPX) **Antidepressants** Carbamazepine (CMP)	SMX: 75-100% TMP: 50-75% ERY: 75-100% ROX: 75-100% TRC: <20% DCL: 75-100% NPX: 75-100% CMP: <50%	Two lab-scale anaerobic digesters/A mixture of primary and secondary sludge	-The controversial results reported for some organic micropollutants reveal a poor understanding of the mechanisms and factors behind their biotransformation. - Temperature is critical in reducing estrogenicity since only thermophilic conditions guarantee that estrogenicity decreases. -Combining chemical and biological methods to characterize the quality of digested sludge in terms of emerging micropollutants is essential to settle operational strategies, such as thermophilic digestion of sludge, that promote a safer disposal.	Gonzalez-Gil et al. (2016)
BES	**Antibiotics** Sulfamethoxazole (SMX)	SMX: 85%	Two-chamber MFC reactors/ Synthetic WW	- A long acclimation period could significantly enhance the degradation ability of microbes towards recalcitrant chemicals. - SMX was rapidly degraded in MFC.	Wang et al. (2016)
	Antibiotics Tetracycline (TC)	TC: 79.1%	Two-chamber MFC reactors/ Synthetic WW	- Synergy of fermentative, acid-producing, and electrogenic bacteria effectively degraded TC. - A gradient acclimation is necessary to achieve higher removal of TC in MFC.	Wang et al. (2017a)
	Antidepressants Carbamazepine (CMP)	CMP: 84%	A dual chamber BES reactor/ Synthetic WW	- A decrease in CMP degradation was observed, which can be attributed to a low number of active bacteria and a poor ability to adapt to high voltage. - Anode potential enhanced the microbial colonization, which stimulated more microbial activity and resulted in an enriched biofilm that targeted CBZ.	Tahir et al. (2019)

Technology	Emerging pollutants	Removal (%)	Process	Remarks	References
BES	**Anti-inflammatory** Diclofenac (DCL) Naproxen (NPX) **Antidepressants** Carbamazepine (CMP)	CW-MEC DCL: 10-17% NPX: 10-17% CW-MEC CMP: 10-17% CW-MFC All compounds: 5%	Constructed wetlands (CWs) operated as BES/ MFC and MEC/ laboratory conditions with synthetic WW	- No statistically significant effect of CW-BES on organic micropollutants removal were found. - An increase of organic micropollutants removal were noted in CW-MEC and CW-MFC when compared to CW-control. - An increase in microbial activity due to the indirect effects of electrolysis-induced increase of aerobic degradation.	Hartl et al. (2021)
AGS	**Antibiotics** Sulfamethoxazole (SMX)	SMX: 98%	Lab- scale AGS-SBR/ Synthetic WW	-High organic load concentration of the influent improved the removal of SMX, favouring granule stability and settling - Increasing SMX concentrations enhanced EPS secretion, particle sizes, and the SMX removal loading rate for various antibiotic concentrations.	Cui et al. (2021)
	Antibiotics Oxytetracycline (OTC)	OTC: 55%	Lab-scale AGS-SBR/ Synthetic WW	- Aerobic granules maintained their structural and functional stability under low OTC without major changes in particle sizes. - The systems reached a steady state and kept excellent COD, NH_4^+-N, and TP removal (over 90%) in the presence of OTC. - A fast adsorption was responsible for the degradation of OTC by AGS.	He et al. (2021)
	Antibiotics Sulfamethoxazole (SMX)	SMX: 90%	Lab-scale AGS-SBR/ Synthetic WW	- SMX had no significant impact on the treatment performance on granular stability. - EPS content remained stable during the experiment.	Kang et al. (2018)
	Antibiotics Trimethoprim (TMP) **Anti-inflammatory** Diclofenac (DCL) Naproxen (NPX) **Antidepressants** Carbamazepine (CMP)	TMP: 50% DCL: 50% NPX: <30% CMP: <30%	Lab-scale AGS-SBR/ Synthetic WW	- TMP was highly resistant to degradation, and the superficial charges of EPS caused the removal mechanism via adsorption in the external layers of the AGS. - CMP was the most recalcitrant compound in the effluent. - The presence of pharmaceuticals made the granules more compact and denser than without drugs.	Muñoz-Palazon et al. (2021)

Consistently, there were low antibiotic removal rates in WW from 16 Turkish hospitals treated by conventional WW treatment, particularly for CTC, ERY, OTC, SMX, sulfamethazine, and TMP (Li et al., 2015a). Similarly, Dinh et al. (2017) found a poor removal level of CIP, ERY, NOR, SMX, TMP, and vancomycin in a French CAS treating hospital and urban WW. There was also a high persistence of amoxicillin and CIP in the effluent generated after CAS treatment of WW from a Kenyan hospital (Kimosop et al., 2016). The removal rate of SMX in another Kenyan WWTP receiving hospital effluent was only 38% on average (Ngigi et al., 2020).

Several studies have reported the removal efficiencies of ARB and ARGs by using CAS treatment. Timraz et al. (2017) found higher removal efficiencies of *tetO* (100% removal), *tetZ* (100% removal), and *intl2* (97% removal). In contrast, these authors described that the *sul*1 and *sul*2 genes increased their abundance by 14% and 155% in the effluents and that the *intl*1 gene was only reduced by 62% in the effluents from two hospitals in Riyadh (Saudi Arabia) compared to the corresponding influents. Rafraf et al. (2016) showed that CAS treatment of WW receiving hospital waste did not reduce the abundance of the *ermB* and *sul*1 genes. After the WW treatment, they found a higher abundance of the $bla_{\text{CTX-M}}$, bla_{TEM}, and *qnrS* genes.

Hence, a plethora of studies have described that inefficient antibiotic removal via CAS systems could result in the activated sludge microbial communities being exposed to continuous sub-minimal antibiotic concentrations. Long-term exposure to sub-minimal inhibitory concentrations of antibiotics and high nutrient loads and biomass production promotes the proliferation of ARB, creating new niches in the activated sludge favourable for the horizontal transfer of ARGs (Guo et al., 2017). Hence, CAS systems have been frequently described as potential hotspots for antimicrobial resistance transmission (Korzeniewska and Harnisz, 2018; Marti et al., 2013a). In addition, the low removal rate of antibiotics usually achieved by CAS technology can generate metabolites and various transformation products with antimicrobial activity, which may be more toxic than the parent compounds in the final effluent (Timraz et al., 2017; Majumder et al., 2019). Considering the worldwide prevalence of the use of CAS systems, their role in disseminating antibiotic resistance cannot be neglected as it is still a vital matter to solve. Therefore, additional studies are required to comprehend better the prevalence and activity of resistant bacteria in WWTPs that use CAS technology. Notably, future research

should investigate the relationships between antibiotic contamination and the role of activated sludge bacterial communities as a reservoir for ARB and ARGs.

1.4.2 Membrane bioreactors

MBR technology has been widely used for emerging pollutant removal, particularly antibiotics from hospital WW (Beier et al., 2012; Prasertkulsak et al., 2016). The MBR procedure combines both biological treatment and physical separation through membranes of the pollutants within one processing unit (Du et al., 2020). Compared with CAS, MBR has several advantages, such as a small environmental footprint, a high solid retention time (SRT), lower sludge production, and high effluent quality (Shi et al., 2018). In addition, the ever-decreasing cost of membrane materials and the increasingly stringent requirements of treated effluent quality have led to the more widespread use of MBR technology (Abdelrasoul et al., 2018). The major concern of the application of MBR technology is its high energy consumption due to membrane fouling (Xiao et al., 2019). Hence, there should be proper control of factors influencing biofouling (Men et al., 2017).

Several studies have reported that MBR technology can produce higher antibiotic removal efficiencies than CAS technology (Zhao et al., 2021b). In particular, removal efficiencies >90% have been described for amoxicillin (Kamaz et al., 2019) and CIP (Do et al., 2018) in MBR-WWTPs. Prasertkulsak et al. (2016) found improved biodegradation of SMX and TMP (around 80% efficiency) in a pilot-scale MBR installed at a hospital in Bangkok (Thailand). However, Kovalova et al. (2012) detected removal efficiencies of <60% for AZN, CMP, CIP, CLM, CCLP, ERY, metronidazole, NOR, SDZ, SMX, and sulfapyridine in six pilot-scale MBR treatment plants placed in Swiss hospitals. These differences could be related to the changes in the operational configuration, which could result in significant differences in the removal efficiency of antibiotics (Reif et al., 2011; Shi et al., 2018). Concerning PhACs removal, poor elimination ratios have been reported for anticancer (CCLP), anti-inflammatory (DCL and NPX), and antidepressant (CMP) drugs (Kovalova et al., 2012; Prasertkulsak et al., 2016).

MBR technology usually operates under a high SRT, favouring the proliferation of more diverse bacterial communities, which may facilitate the removal of antibiotics in MBR by improving their adsorption on the flocs, yielding higher antibiotic biodegradation rates (Gu et al., 2018). Different authors have determined the ARG and ARB removal capacity in MBR (Le et al., 2018; Wen et al., 2018; Zhu et al., 2018); however, the role of MBR

systems receiving hospital WW in the amelioration of the resistance phenomenon has been scarcely addressed. In one study, a pilot-scale hospital MBR system for the *in situ* treatment of hospital WW achieved a total removal of bla_{SHV}, *tetB*, *tetM*, and *vanA*. However, the abundances of these genes were 0.4-1.8-fold higher in the hospital WW compared to the communal samples (Paulus et al., 2019). In addition, Chiemchaisri et al. (2022) recently reported a total removal in the number of isolated ARB, including *E. coli*, *K. pneumoniae*, and *Acinetobacter baumannii* in the effluent from pilot-scale MBR sludge than those of the corresponding hospital WW. However, increased prevalences were found in the MBR sludge compared to the influent for *E. coli* (ratio influent/sludge: 41.6), *K. pneumoniae* (ratio influent/sludge: 16.5), and *A. baumannii* (ratio influent/sludge: 1.46).

In general, the dense biomass layer and the membrane itself are barriers that effectively avoid leakage of ARB from the membrane module to the environment, thus holding back ARGs within the membrane module. However, increasing membrane fouling due to continuous biomass production can change the solubility of microbial products and the excretion of extracellular polymeric substances (EPS), creating a dense biofilm that facilitates the transfer of ARGs to antibiotic-sensitive bacteria (Zhu et al., 2018). Hence, a strategic reduction in membrane biofouling should be considered to minimize the increase in ARB within the biofilm layer (Wen et al., 2018). Finally, it is worth mentioning that the complex reaction conditions of MBR treatment require additional research regarding the simultaneous impact of multiple factors to enhance the use of MBR. In addition, information about the biotransformation products and the effect of dissolved oxygen on antibiotic removal and ARB population reduction via proper MBR operation should be addressed (Gu et al., 2018).

1.4.3 Anaerobic digestion

Sewage sludge treatment is considered one of the most significant issues in WW treatment due to higher energy demands and treatment costs (Yan et al., 2015). Furthermore, a plethora of studies have reported the strong presence of antibiotics and pathogenic microorganisms in these residual solids (Ostman et al., 2017). Hence, sewage sludge is an essential reservoir of antibiotics, ARB, and ARGs, and their improper management could increase the dissemination of ARB and ARGs into the natural environment (Tian et al., 2016; Wu et al., 2018). AD is the most widely used method for the intensive biodegradation of sewage organic fractions derived from municipal and industrial solid

wastes (Adekunle and Okolie, 2015; Jang et al., 2020) owing to its excellent capacities for mass reduction and energy recovery via hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Zhang et al., 2021). Besides, AD is a proven and even preferred method for sludge stabilization from WWTPs. Thus, it is used to disinfect pathogenic organisms and other unwanted contaminants of the sludge (Gonzalez-Gil et al., 2016; Wu et al., 2018).

Antibiotics, ARB, and ARG removal by AD have been mainly tested for swine manure and actual WW; however, a few reports have addressed AD application in hospital WW treatment (Langbehn et al., 2021). In general, the removal of antibiotics in sewage sludge occurs via sludge sorption, followed by rapid sludge desorption, and then biodegradation. Numerous experimental studies have demonstrated that AD treatment only has moderate and variable antibiotic removal rates (40%-77%) (Feng et al., 2017; Yin et al., 2018; Visca et al., 2021). Besides, most ARB and ARGs cannot be removed entirely through AD of sewage sludge, as a reduction rate of ARG of only 34%, in terms of richness, was found during the process (Ju et al., 2016). However, a physical or chemical pretreatment step before digestion can improve ARB and ARG removal by nearly 50% (Tong et al., 2017).

Similarly to what is observed in CAS systems, exposure to subminimal antibiotic concentrations can affect the microbial community associated with AD sludge. Also, according to Xiao et al. (2021), several factors, mainly the temperature, influence the degree of antibiotic removal by AD. Regarding the effect of temperature on the removal of ARGs, Zhang et al. (2021) recently revealed that the abundance of plasmids carrying different ARGs was significantly lower in thermophilic AD sludge operated at 55˚C than that in mesophilic digesters (25˚C and 35˚C). According to these authors, this difference is due to the replacement of the ARG enriched Bacteroidetes and Firmicutes phyla to the *Thermotogota* phylum, which carry lower ARG determinants. Hence, high-temperature AD is more conducive to controlling ARGs in sewage sludge. In this trend, temperature was the main factor controlling the removal of PhACs in the AD dual reactor treating a mixture of primary and secondary sludge.

Considering the available data, AD could achieve strong antibiotic removal and inhibition of potential ARG-harboring hosts; however, some aspects have been scarcely studied. Xiao et al. (2021) highlighted that the influence of mixed antibiotic effluents, the type of

sludge substrates, the structure of microbial communities, and the kinetic adsorption of antibiotics should receive additional research attention.

1.4.4 Bioelectrochemical systems

BES in WW treatment is a novel approach used for different purposes such as energy generation, remediation of heavy metals/toxic compounds, water desalination, and synthesis of value-added products. BES clearly stands out for its ability to use microbial metabolism to produce chemical energy from biodegradable materials (Logan et al., 2019). These systems display different arrangements in WWTP schemes, using biological electrodes or a combination of biological and chemical electrodes as microbial fuel cells (MFCs) and microbial electrolysis cells (MECs) (Kelly and He, 2014; Zhang et al., 2020b). Compared with CAS systems, one of the most attractive advantages of BES is the low sludge production (Hassan et al., 2021).

MFCs remove antibiotics in a two-chambered configuration, in which improved mineralization is achieved due to the combination of anaerobic biodegradation and electrical stimulation by the anode chamber (Wang et al., 2018a). In MFC systems, antibiotics are mainly used as electron donors; they play an active role as carbon and nitrogen sources (Wang et al., 2016b). Researchers have demonstrated the utility of MFCs in antibiotic degradation: Ceftriaxone was biodegraded at a rate of 91% (Wen et al., 2011), and chloramphenicol was removed at a rate of 97% (Zhang et al., 2017a). Interestingly, another study emphasized the importance of gradient acclimation in the process of antibiotic removal by MFCs (Wang et al., 2017a). Besides, recent evidence from lab-scale experiments has indicated a lower relative abundance of ARGs in effluent generated by MFC technology than that of conventional bioprocesses (CAS and AD) during long-term operations (Hassan et al., 2021). In this regard, the removal efficiencies of beta-lactam *bla*$_{\text{OXA-1}}$, *bla*$_{\text{OXA-2}}$, and *bla*$_{\text{OXA-10}}$ genes in a BES system were 99.3%, 99.8%, and 99.9% (Cheng et al., 2016), highlighting the potential capacity of MFC in the treatment of WW enriched in ARGs. However, the use of BES technology for the removal of PhACs has been limited, and poor removal rates have also been reported. According to Hartl et al. 2021, extremely low values (<17%) were observed for DCL, NPX, and CMP.

It should also be noted that the BES technology combined with other biological treatments makes better WW treatment feasible, ameliorating some disadvantages of conventional bioprocesses (Ghangrekar et al., 2020). Integrating BES and MBR systems reduces

membrane fouling because biodegradation and electrochemical and membrane filtration processes are combined into one unit, providing higher effluent quality compared with conventional MBR and CAS (Ensano et al., 2016).

Despite some experimental evidence in the literature regarding antibiotics, ARB, and ARGs removal by BES, its role in mitigating the antibiotic-resistance phenomenon has been scarcely investigated. Among the aspects that should be clarified, two stand out: the effect of electrical stimulation on the physical properties of bacteria (transmembrane permeability and membrane potential) (Zhang et al., 2020b) and the influence of operating parameters on the occurrence and abundance of ARB and ARGs (Wang et al., 2018a; Yan et al., 2019). Besides, the use of BES at an industrial scale has been hindered, and the ability to scale-up needs to be improved before there can be real-scale implementation of MFC reactors (Langbehn et al., 2021).

1.4.5 Aerobic granular sludge systems in sequencing batch reactors

AGS-based engineered systems under sequencing batch technology are regarded as one of the most promising WW treatment technologies (Nancharaiah and Kiran Kumar Reddy, 2018). Granular biomasses are spherical sludge aggregates composed of self-immobilized microorganisms embedded in an EPS matrix (Oliveira et al., 2021). The microbial communities of granular flocs are composed of heterotrophic and nitrifying bacteria in aerobic zones, phosphate-accumulating organisms and glycogen-accumulating organisms in interlayers (microaerobic zone), and denitrifiers in the core of granules (anaerobic layer) (Ji et al., 2021). This design provides several metabolic pathways from which bacteria can obtain energy, which is possible due to the coexistence of aerobic, microaerobic, and anaerobic layers within the granule (Barrios-Hernández et al., 2021). Therefore, AGS biomass is highly distinguishable from floc biomass found in CAS or MBR systems. Moreover, AGS systems are generally operated as sequencing batch reactors (SBRs), a fill-and-draw-activated sludge system in which WW is added to a single batch reactor, discharging clean water after eliminating undesirable components (Al-Rekabi et al., 2007). Hence, merging both technologies into AGS systems results in all metabolic reactions and solid-liquid separation in a single tank under a continuously repeated time sequence (Singh and Srivastava, 2011).

Compared with conventional WW treatment, AGS systems exhibit several advantages, mainly a simple structure, flexible operation methods, a small footprint, reduced

operational costs, and an additional sludge recirculation system is not required (Huang et al., 2022). In addition, the use of granular biomass stands out due to the excellent sludge settlement; high carbon, nitrogen, and phosphorus removals in the same unit; strong resistance to shocks of toxic compounds; high biomass retention; and low or null production of waste biomass (Lv et al., 2014; Abbass et al., 2018). It is generally assumed that WW treatment based on AGS systems is reliable, cost-effective, and highly efficient, and it is already helping to answer the current worldwide WW treatment challenge. Indeed, AGS leads to approximately 25% cost reduction and around 75% lower surface area requirement (Kehrein et al., 2020). In addition, the low demand for chemical compounds makes AGS a promising technology in WW treatment, including industrial WW (Hamza et al., 2021). A summary of the functional capabilities of AGS system and its main biological mechanism for contaminant removal is shown in Figure 4.

Despite the very limited research addressing the effectiveness of AGS technology for antibiotic removal, different studies have demonstrated the feasibility of this innovative technology for antibiotic removal from WW; however, varying removal efficiencies have been reported. The removal rates of SMX and NOR were only around 60% in AGS-treated influents supplemented with 50 $\mu g\ L^{-1}$ of each therapeutic compound, independently of the maturation stage of the granule (Zhao et al., 2015). Similarly, the removal rates of TC, OTC, CTC, and SMX were around 75% in different AGS systems fed with synthetic WW supplemented with 50 $\mu g\ L^{-1}$ of TC or 3 $mg\ L^{-1}$ of SMX (Liu et al., 2019b; Cui et al., 2021).

On the other hand, raw piggery waste treated through a lab-scale AGS system showed a total antibiotic removal rate near 90%; kanamycin and TC were removed most efficiently (Wang et al., 2019). Wang et al. (2017b) obtained a similar result for ampicillin (AMP) in a lab-scale AGS system (97% removal efficiency) when using different concentrations (5, 10, and 15 $mg\ L^{-1}$). Remarkably, the organic loading rate is a key operational parameter in the elimination of sulfonamides, as the removal efficiency increased from 82% to 90% as the biomass concentration increased from 6.4 to 11.8 $g\ L^{-1}$ in a lab-scale AGS system fed with synthetic swine WW (Liu et al., 2019b). Regarding PhACs removal by AGS systems, more studies need to be conducted as only a few reports have evaluated the PhACs removal in AGS systems, and mostly at laboratory scale using synthetic WW for DCL, NPX and CMP moderate removal rates around 50% have been reported (Muñoz-Palazon et al., 2021b).

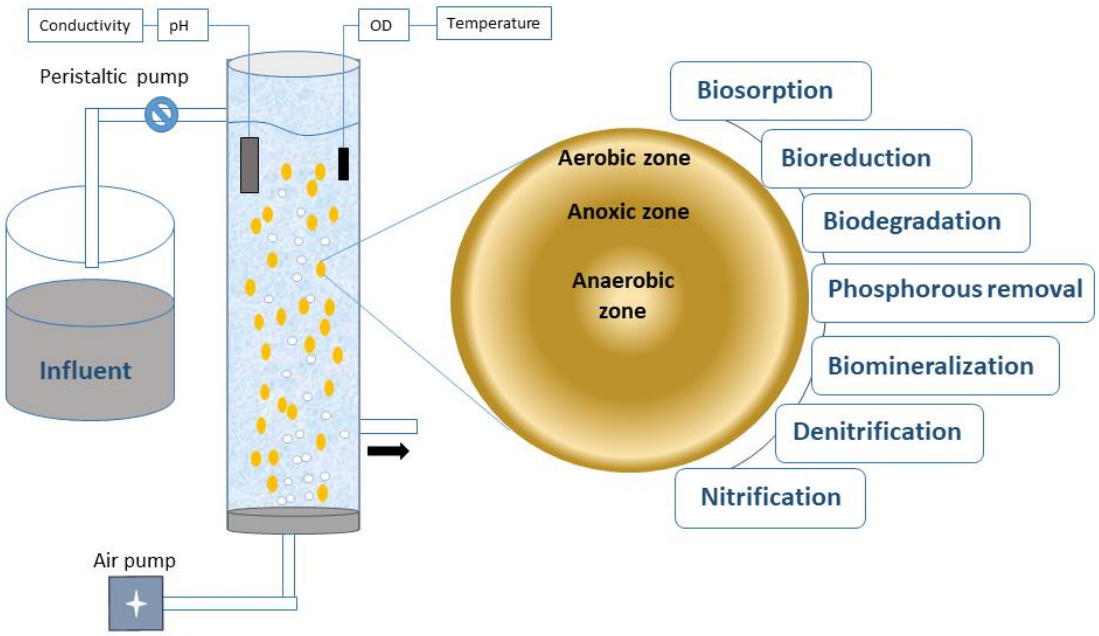


Figure 4. Schematic diagram of the functional capabilities of aerobic granular sludge (AGS).

Antibiotics are also a key factor driving the microbial community structures, as high antibiotic concentrations could usually result in shifts in bacterial communities of AGS. For example, 1000 μg L^{-1} of SDZ in the influent decreased the microbial diversity but enriched the sulfonamide resistance genes (*sul*1 and *sul*2) (Wang et al., 2022). Similarly, the abundance of the *aac* (6′)-Ib *ermB*, *strB*, *sul*1, *sul*2, and *tetX* genes was positively correlated with higher biomass content determined by the mixed liquor suspended solids (MLSS) and diameters of the granules after 71 days of operation in an AGS system inoculated with different types of seed sludge (Li et al., 2020b). In addition, the different feeding modes of WW within an AGS system have broad effects on the formation of granular biomass, causing knock-on effects of variations in the abundance of ARGs due to the complex relationships between the changing size of sludge and the microbial communities (Guo et al., 2023). Mainly, the feeding of the influent introduced uniformly through the bottom of the SBR configuration resulted in rapid growth of ARB but diminished the abundance of ARGs after the achievement of a stable granulation. However, an inlet WW flowing directly from the top failed to cultivate granular biomass, and higher abundances of ARGs were found in this system compared to the ones independently of the ARG.

Despite the influence of antibiotics on granular biomass properties and structural stability, various studies have demonstrated that biomasses exposed to different antibiotic concentrations, such as 10 mg $L^{-1}$of AMP (Wang et al., 2017b), 2 µg L^{-1} of SMX (Kang et al., 2018a), or 900 µg L^{-1} of OFL (Shi et al., 2021) were stable under these conditions. However, other authors have highlighted that prolonged, high-level exposure to fluoroquinolones, cephalexin, or SDZ resulted in worse performance of the AGS technology (Amorim et al., 2014; Wang et al., 2022). Strikingly, Ren et al. (2023) have recently described that different layers of the granules exhibited different behaviours in the ARGs concentration, as the outer layer owned the most abundant gene *sul*1 while the inner AGS owned the least, despite a total increase of 3.2 times in the *sul*1 gene copies of the AGS after 164 days of operation compared to the original inoculum.

Overall, antibiotic removal carried out by AGS reactors can be considered satisfactory; however, the bacterial communities developed on the granules can act as AGS resistance hotspots. In this regard, Sabri et al. (2020) found removal rates of 99.99%, 99.97%, 99.99%, and 100.00% for the *ermB*, *sul*1, *sul*2, and *tetW* genes, respectively, and only an enrichment in the biomass of the granule of the *ermB* gene (2.76 times) compared to that of the influent. Similarly, Wang et al. (2019) found, after 155 days of operation, removal rates of bacteria resistant to AMP, ERY, TC, kanamycin, and CIP of 94.74%, 95.19%, 92.86%, 96.00%, and 93.54%, respectively; however, a broad enrichment in the ARB within granular biomass was also found (average of 18 times higher abundances in the AGS compared to those of the influent). This could be because the high biomass within the AGS provides more microbial contact events, leading to higher ARG transfer within the bacterial communities of the granules (Yang et al., 2013). Furthermore, the long retention times often used in AGS systems could enhance the antibiotic removal ability and increase the risk of a higher abundance of ARGs (Liao et al., 2019b).

Future research directions should pursue the integration of AGS with other existing WW treatment technologies (Kehrein et al., 2020; Stes et al., 2021) and operation in continuous-flow AGS rectors (Li et al., 2019; Meng et al., 2020). Furthermore, it should be mentioned that the current general WW treatment strategies proposed by government authorities tend to integrate different technologies to refine WW management, improving techno-economic impacts and lowering environmental impacts (Ghangrekar et al., 2020). For example, one promising strategy is to combine AGS technology in MBR systems to reduce biofouling, which has shown excellent potential for antibiotic removal (78% and

88% of SMX and NOR, respectively (Xia et al., 2015). On the other hand, recent studies have pointed out that AGS rectors operated in continuous-flow mode could enhance the loss of stability in long-term operations using SBRs (Rosa-Masegosa et al., 2021). Nevertheless, the effect of antibiotics on the AGS in continuous-flow performance has not been determined. Therefore, additional research is necessary to minimize the enrichment of ARGs in the bacterial communities of granular sludge biomass. The role of granules as a reservoir of pathogenic microorganisms in the face of ARG-enriched environments and the effect on the microbial community structure found in the granular biomass is not well-known and must also be elucidated. Finally, further investigation of bacterial community dynamics and evaluation of the genetic resistome is required to understand the operation of these novel WWTP configurations.

1.5 PhACs removal mechanisms for the selected treatment biotechnologies

Microbial processes via degradation of PhACs, either by metabolic processes or co-metabolism and sorption on sludge flocs and microbial surfaces, are among the biological reactions for pollutant removal from WW. Several parameters and constraints affecting the removal of PhACs have been identified, including the physicochemical properties of the compounds and operating parameters such as influent concentration, seasonal variation, hydraulic retention time (HRT), and sludge retention time (SRT). In particular, the elimination mechanisms of antibiotics have been well described. Antibiotics may undergo several transformations by various mechanisms and pathways during WW treatment, including biodegradation, sorption, dispersion, dilution, photodegradation, and volatilization (Rout et al., 2021). Mechanisms that must be enhanced within the WW treatment to ameliorate the negative effect of the high presence of antibiotic residues in WW, particularly hospital WW. Adsorption and degradation are the major antibiotic removal mechanisms in WW treatment, independent of the technology employed and the type and concentration of antibiotics (Azuma et al., 2018). However, the removal pathways are poorly defined and may vary according to the target antibiotic.

1.5.1 Antibiotic adsorption

Antibiotic adsorption involves their accumulation from the water phase to the surface of the solid-phase sorbent either by physical or chemical phenomena (Wang and Wang, 2015). Electrostatic interactions (cation and anion attraction), hydrophobic interactions, cation exchange, cation bridging, surface complexation, and hydrogen bonding are the

most important ways antibiotics are adsorbed onto sludge (Oberoi et al., 2019). Numerous studies have demonstrated that the adsorption process is highly complex and depends on the sorption coefficient (*Kd*), which is directly related to the physicochemical properties of the sorbate (antibiotics) and the sorbent (activated sludge): specific surface area, porosity (macro or microporosity), pore diameter, and functional groups of the molecule (Ferreira et al., 2016; Zhu et al., 2021). In this sense, EPS produced by microorganisms is the key component involved in the adsorption of antibiotics onto the activated sludge (Yu, 2020), which contain abundant and diverse functional groups (amine, carboxyl, and hydroxyl groups, or EPS-bound metal ions) that facilitate their adsorption (Oberoi and Philip, 2018). Several antibiotics can be effectively adsorbed onto EPS, such as CIP, ERY, NOR, and TC (Métivier et al., 2013; Xu et al., 2013; Song et al., 2014; Wang et al., 2018b; Zhang et al., 2018). However, the adsorption of sulfonamides by activated sludge particles is negligible in accordance with their low sorption coefficient (Thiele-Bruhn, 2003).

Numerous variables, including pH, temperature, ionic strength, the types and concentrations of antibiotics, and the types of sludge systems, affect the degree of sorption of these therapeutic compounds to EPS (Cycon et al., 2019). A higher mass ratio of proteins/polysaccharides in EPS results in stronger hydrophobicity, greater availability of adsorption sites, and higher binding strength, factors that increase under higher antibiotic concentrations (Zhang et al., 2018). Compared with the conventional bioprocess (CAS or MBR technologies), EPS produced from AGS contain a higher protein-polysaccharide proportion, resulting in stronger hydrophobicity and more sites for antibiotic adsorption (Oberoi et al., 2019; Zhu et al., 2021). In addition, many functional groups (carboxyl, hydroxyl, amine, amide, sulfhydryl, and aldehyde) are commonly present on the EPS polysaccharides and proteins on the AGS surface. Hence, increased antibiotic, ARB, and ARG elimination can be achieved by using these systems (Nancharaiah and Kiran Kumar Reddy, 2018).

1.5.2 Abiotic and biotic degradation of antibiotics

Antibiotic biodegradation involves their hydrolytic breakdown by modification (biotransformation), cleavage (biodegradation), or mineralization to CO_2 and H_2O (Reis et al., 2020). A plethora of metabolic mechanisms mediate the biotransformation of antibiotics as microorganisms utilize these compounds as carbon and energy sources (Oberoi et al., 2019). In addition, some antibiotics can be abiotically degraded,

highlighting the photochemical transformation as the core process by which these compounds are abiotically degraded (Conde-Cid et al., 2018).

Among aminoglycosides, gentamicin and kanamycin are abiotically degraded in natural matrices at higher temperatures and low pH (Cara et al., 2013). On the contrary, aminoglycosides are generally considered non-biodegradable by microorganisms in both aerobic and anaerobic conditions in activated sludges (Gartiser et al., 2007). Chloramphenicol and florfenicol can be abiotically modified by hydroxylation, dichlorination, and photolysis (Xue et al., 2021). Amphenicol biodegradation is based on dichlorination and demethylation reactions, producing metabolites with increased persistence in WW compared with the parent molecule (Reis et al., 2020). Beta-lactams have inherent instability due to their four-membered lactam ring, which is readily hydrolyzed both abiotically and enzymatically. However, these metabolites are more recalcitrant than the original drugs, as they are often found in WW effluents (Zumstein and Helbling, 2019).

Regarding their biodegradation, beta-lactamases are the main enzymes that hydrolytically act over these compounds, which are differentiated concerning the type of target beta-lactam substrates: penicillins, cephalosporins, carbapenems, or monobactams. It is worth noting that the degradation of beta-lactams is intimately associated with beta-lactam resistance, and antibiotic-degrading bacteria have the potential to propagate this resistance to susceptible bacteria (van Hoek et al., 2011). Macrolides are photolytically removed by solar light, forming a large variety of by-products, a process encouraged by higher concentrations of dissolved organic carbon (Lange et al., 2006). In contrast, some studies have indicated that macrolide biodegradation is incomplete in conventional WWTPs and MBRs (Ibanez et al., 2017; Senta et al., 2017). Quinolones are extensively transformed by photolysis and photo-catalytic processes; nevertheless, the intermediates are not entirely eliminated because the quinolone core structure remains intact after their abiotic degradation (Tang et al., 2019). In addition, fluoroquinolone biodegradation is a slow, non-dominant process in microbial communities, suggesting the recalcitration of these therapeutic compounds (Zou et al., 2022). Nonetheless, some specific transformations of ENR, NOR, OFL, and danofloxacin have been described (Cvancarová et al., 2015). Abiotic degradation of sulfonamides relies on photolysis and hydrolysis (Deng et al., 2018). On the other hand, biotic degradation includes heterocyclic modifications (Ricken et al., 2017). It should be noted that the biotransformation of

sulfonamides depends on nitrogen availability, as SMX metabolism is enhanced under nitrogen starvation (Müller et al., 2013). Tetracyclines are mainly eliminated abiotically due to their reduced stability in WW; the specific pathway depends on the temperature, pH, ionic strength, and chelation with cations (Wu and Fassihi, 2005). Biological transformation plays only a minor role in the removal of tetracyclines (Spielmeyer et al., 2015), as only *Stenotrophomonas maltophilia* has been reported to biodegrade TC (Leng et al., 2016). Finally, TMP is not very susceptible to photolysis (Kim et al., 2015) and biodegradation (Li and Zhang, 2010). However, it is demethylated under nitrogen removal conditions by the action of the ammonia monooxygenases of nitrite-oxidizing bacteria (Men et al., 2017).

In AGS systems, antibiotics undergo several biological metabolic transformations due to the coexistence of aerobic, microaerobic, and anaerobic layers within the granule at the same time (Shi et al., 2021). Hence, the biological degradation of antibiotics can be remarkably strengthened by using granular biomass due to the synergistic effects of the multiple microbes in the metabolic pathways of antibiotics (Barrios-Hernández et al., 2021).

1.6 Future research directions

Among organic micropollutants, PhACs have been extensively investigated due to their potential adverse ecological and human health effects. WWTPs are the primary barriers that prevent them from reaching sensible ecosystems. However, CAS systems are only partially effective in removing and degrading PhACs, especially for toxic and recalcitrant antibiotic compounds. In addition, the alternative bioprocesses evaluated for PhAC elimination have shown several limitations, especially for hospital and healthcare system WW treatment. Over the recent decades, AGS has been highlighted as a universal replacement for CAS, with encouraging results for PhAC enrich-wastewater treatment due to the complex microbial metabolism within the granular biomass.

Although many efforts have been made to control the spread of antibiotic resistance to the environment, some essential concerns should be resolved, and significant knowledge gaps must be bridged. In this respect, *i*) there is insufficient information about the role of hospital WW as a central reservoir of ARB and ARGs, *ii*) the lack of proper regulation to guide the limit of antibiotics, ARB, and ARGs before their discharge into public sewers and receiving water bodies, *iii*) novel ARB and ARGs need to be easily monitored in

WWTPs, remaining biotechnologies and natural environments, mainly through the implantation of standardized qPCR protocols, *iv*) the disclosed AGS as the technology of choice needs to be optimized to achieve higher antibiotic removal rates, limiting the proliferation of ARBs and ARGs. This endeavour will allow the development of better strategies to control the spread of antibiotic resistance, especially from WWTPs and healthcare facilities to the rest of the environment.

2

2-OBJECTIVES

In general, CAS systems that co-treat wastewater from both hospital and municipal sources promote the spread and proliferation of ARB and ARGs in the receiving aquatic ecosystems. Hence, the search for novel bioprocesses providing a reliable solution to avoid the amplification of the resistance phenomenon is an urgent matter. AGS systems are currently considered as the most promising technological alternative to overcome the drawbacks of CAS; however, despite their satisfactory performance in terms of pollutant removal and stable microbial community composition, the functionality of this technology in the amelioration of antibiotic resistance emergence has been scarcely addressed. Therefore, the impact of real hospital WW influents on the AGS technology in terms of treatment performance, microbiome dynamics, and occurrence and abundance of ARGs needs to be thoroughly investigated. To achieve these goals, the design and validation of new molecular tools, allowing a more reliable evaluation of the biodiversity of ARGs and their quantification through more accurate, sensitive and robust qPCR assays, must be also implemented. Finally, the widespread application of these quantitative studies for the investigation of the distribution patterns of ARGs in WWTP-related environments, particularly in activated sludge biomasses operating under cold temperatures, could contribute to understand the role of WWTPs as potential antimicrobial resistance hotspots.

In accordance with the above, the main objective pursued in this thesis is to investigate the feasibility of using an AGS reactor as an alternative *in situ* technology for the treatment of real hospital WW, aiming to significantly reduce the concentration of emerging contaminants, especially antibiotic compounds, and the proliferation of ARGs and their potential hosts.

For this purpose, the following several partial objectives have been established:

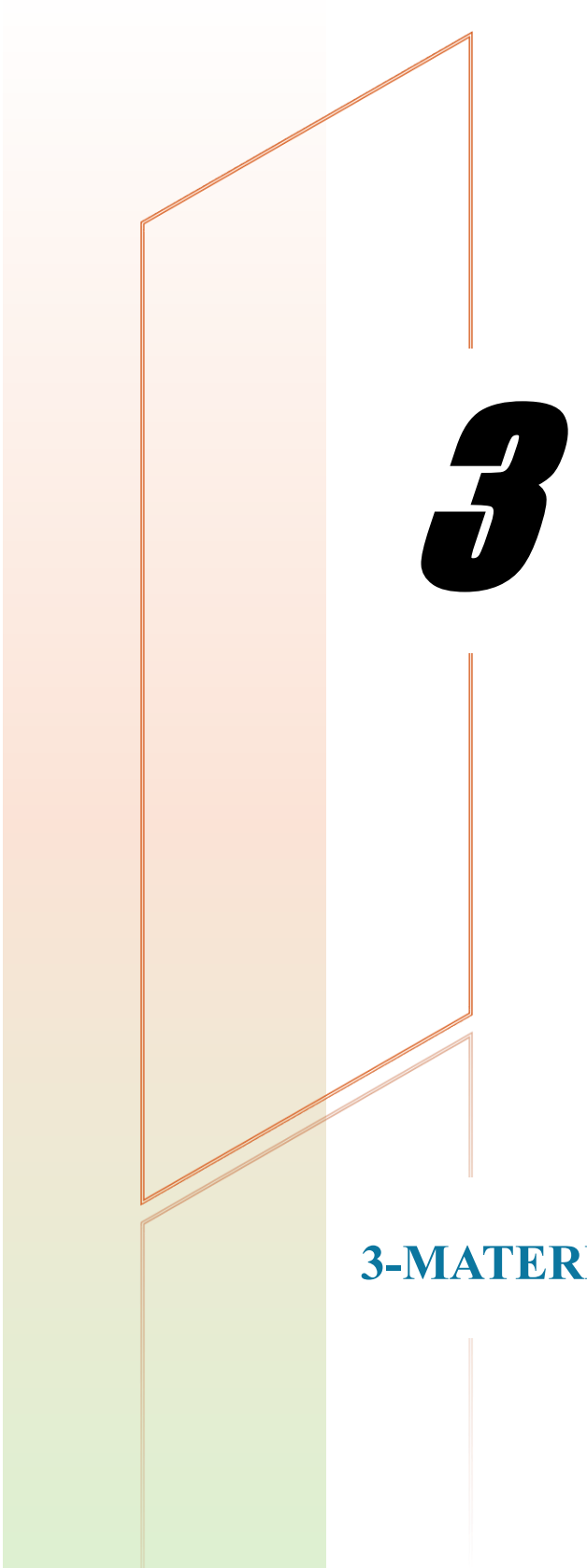
1. To evaluate the stability of the granular biomass and the performance for the removal of common pollutants and clinically relevant PhACs in a lab-scale AGS reactor treating real hospital WW.

2. To determine the impact of the emerging contaminants occurring in real hospital WW, mainly antimicrobials and other PhACs, on microbiome dynamics, focusing on the abundance and diversity of bacterial, archaeal and fungal populations.

3. To develop accurate molecular tools for the quantification of relevant ARGs in WWTP-related samples based on the design of new sets of primers and the validation of qPCR protocols.

4. To characterize the dynamics of the profile of ARGs in an AGS reactor treating real hospital WW, and to investigate the prevalence and abundance of ARGs in the granular biomass throughout AGS operation from start-up to steady-state.

5. To determine the distribution patterns of ARGs in WWTP settings in cold regions to elucidate their role as reservoirs of antimicrobial resistance under these extreme conditions.



3

3-MATERIALS AND METHODS

3. General workflow

The general workflow of the present thesis was based on the use of AGS treating real hospital wastewater (WW), with a particular focus on *i*) AGS system performance, *ii*) microbiome evolution analysis, *iii*) ARG profile determination. The specific tasks are shown in Figure 1.

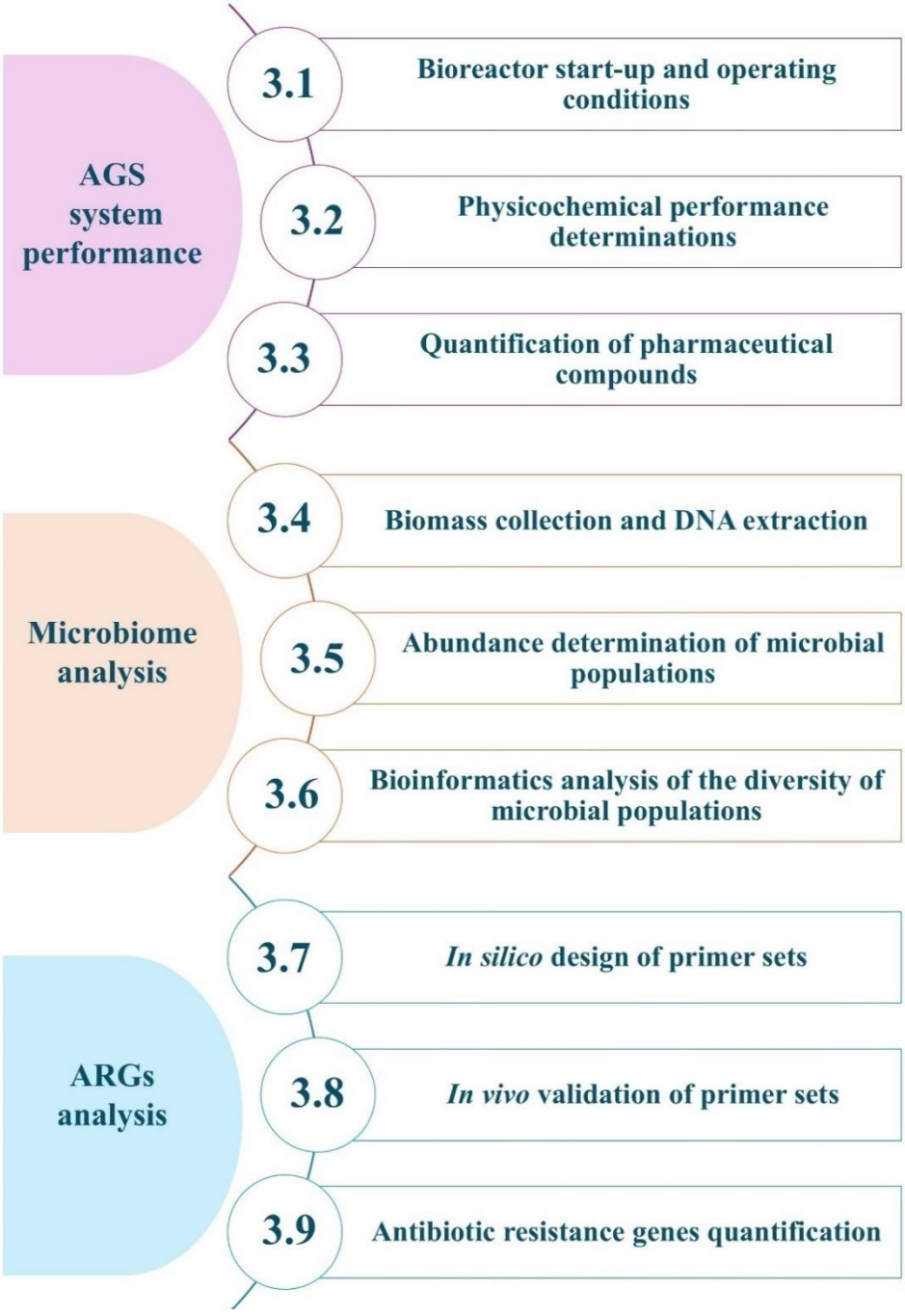


Figure 1. General scheme of the used in this study for the evaluation of AGS treating real hospital wastewater.

3.1 Bioreactor start-up and operating conditions

An AGS system was designed as a cylindrical column with a height of 90 cm and an inner diameter of 7 cm, with a total operating volume of 3 L and operated for 150 days. For the inoculation of the sequencing batch bioreactor, 1 L of granular biomass cultivated with synthetic WW at the laboratory scale was used. Air was supplied at the bottom of the reactor with an air flow of 4.0 L min^{-1} through fine bubbles. The pH, temperature and oxygen parameters were measured periodically throughout the experiment. The effluent flow exchanged 50% of the water volume. The batch cycles consisted of raw water filling (4 min), aeration (170 min), decanting step (3 min) and effluent discharge (3 min). Each cycle lasted 3 hours, and the hydraulic retention time (HRT) was 6 hours. The reactor was fed with a Watson Marlow (United Kingdom) peristaltic pump. The influent was raw water collected from the pipe network of a hospital in Granada (Spain). The raw water was collected once a week at the same time window (every Wednesday from 8.00 to 9.00 a.m.).

3.2 Physicochemical performance determinations

Chemical oxygen demand (COD), BOD on day 5 (BOD_5), and biomass concentration estimated as mixed liquor suspended solids (MLSS) were measured periodically in the influent and effluent water according to the standard methods for the examination of water and WW (APHA, 2012). Samples of raw influent, effluent, and biomass were collected for analytical procedures at days 0, 7, 15, 30, 45, 60, 90, 120, 140, and 150. The stability and properties of the granular sludge were analyzed by the mean size and the settling time according to the methodologies conducted by Muñoz-Palazon et al. (2020a) and Hurtado-Martinez et al. (2021). The concentration of nitrogen ions (NH_4^+, NO_3^-, NO_2^-) and phosphorus anion (PO_3^{4-}) was analyzed using ion chromatography following Muñoz-Palazon et al. (2021).

3.3 Quantification of pharmaceutical compounds

Seventeen drugs belonging to different therapeutic classes were selected for their detection and quantification (ng L^{-1}) in real hospital WW samples. The following antibiotic compounds were included: gentamicin and streptomycin (aminoglycoside class) (Gartiser et al., 2007); ampicillin and amoxicillin (beta-lactam group) (Khan et al., 2021; Langbehn et al., 2021); azithromycin, clarithromycin and erythromycin (macrolides) (Azuma et al., 2018); doxycycline (tetracyclines); sulfamethoxazole

(sulfonamide class) (Al Qarni et al., 2016; Cui et al., 2021); ciprofloxacin (quinolones) (Al Qarni et al., 2016) and trimethoprim (TMP, pyrimidine group) (Muñoz-Palazon et al., 2021b) and were selected due to their high prevalence and distribution in WWTPs and hospital effluents. The remaining drugs were as follows: carbamazepine (anticonvulsant) (Azuma et al., 2018); cyclophosphamide, triclosan (antitumoral) (Al Qarni et al., 2016; Azuma et al., 2018) and ketoprofen, diclofenac, and naproxen (anti-inflammatory) (Prasertkulsak et al., 2016). These drugs were selected due to their high environmental-risk (Muñoz-Palazon et al., 2022a; Olicón-Hernández et al., 2021) and their prevalence in WWTPs environments in previous studies (Wang et al., 2020; Nguyen et al., 2021).

Solid phase extraction (SPE) was used as the pretreatment method (Oasis HLB cartridges, 200 mg). HLB cartridges were conditioned with 8 mL methanol and 8 mL Milli-Q water. Subsequently, the samples (100 mL) were passed through the cartridges. Finally, cartridges were washed with 10 mL Milli-Q water and air dried. Elution was performed with 2 mL methanol, according to Rueda-Márquez et al. (2021). All chromatographic determinations were performed in triplicate using a UHPLC-Q-TOF (Agilent LC 1260 Infinity II coupled with Q-TOF 6470 LC/TQ) to determine the concentration in the influent and effluent samples in the facilities of the Centre for Scientific Instrumentation (University of Granada/Spain). A column Zorbax Eclipse plus C18 - 3x50mm - 1.8 um was used. All PhACs and analytical standards were of high purity and were purchased from Sigma Aldrich (St. Louis, MO, USA $\geq$ 98% purity). Interpolation of the corresponding standard curves was used to determine the concentration of the PhACs. A linearity of 99.99% was achieved in all cases. The standard curve ranges varied from 0.1 ng mL^{-1} to 500 ng mL^{-1}.

3.4 Biomass collection and DNA extraction

Total DNA extraction was performed in several biological samples: Granular biomass from AGS treating real hospital wastewater (Chapter I and III), , activated sludge from two different biofactories in Granada (Spain), composting sludge from sewage sludge composting in the facilities of the Environmental Complex EIDER Recycling Eco-industry (Granada / Spain), anaerobic digestion sludge from the Instituto de la Grasa (Seville, Spain), river sediment from the Genil River in the city of Granada, Spain, agricultural soil from an agricultural field irrigated by the Genil River (Chapter II) and influent and activated sludge from five WWTPs (Finland) (Chapter IV). Solid and

influent samples were centrifuged at 5000 rpm for 20 min at 4ºC, the supernatants were discarded, and the pellets were stored at -20ºC for further use. Total DNA was extracted from 0.5 g of these pellet samples using the FastDNA SPIN kit for soil and the FastPrep 24 instrument (MP Biomedicals, Germany) as described by Correa-Galeote et al. (2021). The quality of the extracted DNA was checked by electrophoresis on 1% agarose and quantified spectrophotometrically at 260 nm using a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, USA).

3.5 Abundance determination of microbial populations

The qPCR assays were performed using DNA extracted from biological samples on Quant Studio™ 3 real-time PCR instruments (Applied Biosystems, USA). Bacterial and Archaeal 16S rRNA genes and 18S rRNA were used as proxies for the total abundance of *Bacteria*, *Archaea*, and *Fungi* using primers 341 F-534 R (Muyzer et al., 1993), ARCH915R-UNI-b-revF (Yu et al., 2008), and FungiQuantF-FungiQuantR (Liu et al., 2012); respectively. Ammonia oxidation and nitrate denitrification populations were also targeted using the *amoA* (Rotthauwe and Witzel, 1997) and *nosZ* genes (Henry et al., 2006), respectively. For phosphate accumulators, 16S rRNA from *Candidatus Accumulibacter* was used as a proxy for phosphate-accumulating organisms (PAO) (Correa-Galeote et al., 2021). In order to study the community structure of microbial populations, the DNA pools were subjected to next-generation sequencing using the MiSeq Illumina platform. The following pair of primers were used to amplify the duplicate DNA samples: Bacteria807F (5′-GGATTAGATACCCBRGTAGTC-3′) and Bacteria1050R (5′-TAGYTGDCGACRRCCRTGCA-3′), for amplification of the V5-V6 hypervariable regions of 16S rRNA from *Bacteria* (Bohorquez et al., 2012), ITS1S (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2S (5′-GCTGCGTTCTTCATCGATG-3′) for *Fungi* (Yan et al., 2023). For the amplification of 16S rRNA from *Archaea*, the following primers were used: 519SGF-(5′- CAGCMGCCGCGGGTAA-3′) and 907SGR (5′- CCCCGTCAATTCCTTTGAGTTT-3′) (Yu et al., 2017).

3.6 Bioinformatics analysis of the diversity of microbial populations

Raw data from the next-generation sequences were subjected to a quality trimming analysis to retain the high-quality reads using the open-access software MOTHUR (Schloss et al., 2009). The remaining reads of archaea and bacteria with incorrect alignment at the forward and reverse position to the SILVA seed database were eliminated

using the Needleman criteria. For all targets, the VSEARCH algorithm was used to detect and remove chimeric reads and discard reads belonging to nonfocus target genes. The clusters of sequences were calculated using the abundance-based greedy method with a similarity of 97% for the bacteria and 95% for the fungi and archaea. The results of the taxonomic consensus in terms of operational taxonomic units (OTUs) were associated with the SILVA_nr data for the *Archaea* and *Bacteria* and with UNITE for the *Fungi*. To construct the OTU tables, singleton OTUs were finally eliminated (Hurtado-Martinez et al., 2021).

3.7 *In silico* design of primer sets for ARGs quantification

Eleven relevant ARGs were selected as targets, including *aadA* and *aadB* (aminoglycoside resistance), *ampC*, *bla*$_{TEM}$, *bla*$_{SHV}$ and *mecA* (beta-lactam resistance), *dfrA*1 (SMX-TMP resistance), *ermB* (macrolide resistance), *fosA* (fosfomycin resistance), *qnrS* (quinolone resistance) and *tetA*(A) (tetracyclines resistance). The *in silico* design of the new primer sets was performed based on the alignment of all sequences of the target ARG (orthology grade >70%) deposited in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, allowing a higher coverage of ARGs biodiversity than several previously described primers. The design of primers optimized the following quality properties: primer size, percentage of guanine and cytosine, lack of formation of secondary structures, and an adequate range of annealing temperatures (Bustin, 2009; Bustin and Huggett, 2017; Dreier et al., 2020).

3.8 *In vivo* validation of primer sets and qPCR protocols

The new molecular tools were validated *in vivo* in six samples: activated sludge, composting sludge, anaerobic digestion sludge, agricultural soil and river sediment. The total DNA extraction was performance as specified in section 3.4. Experimental conditions, thermal profiles, efficiency testing and data normalization were fully achieved according to reference protocols and guidelines (Bustin et al., 2009; Bustin and Huggett, 2017; Dreier et al., 2020).

3.9 ARGs quantification in samples of the study

Quantification studies of ARGs were performed using the pool of total DNA extracted from biological samples, as described in section 3.4. on Quant Studio™ 3 real-time PCR instruments (Applied Biosystems, USA). The specific reaction conditions were carried

out according to the previously developed optimization qPCR thermocycling profiles and plasmids standards construction for each of the targeted ARGs using SYBR-Green I as a day monitoring method (Thermo Scientific, USA). The total copies of ARGs were expressed per gram of environmental sample and normalized as the ratio of the number of ARG copies to the corresponding number of genes encoding 16S rRNA copies to the corresponding number of 16S rRNA coding genes copies, which were determined in all samples according to a previously described protocol (Correa-Galeote et al., 2021).

3.10 Statistical analysis

All experiments were performed in triplicate, and data are presented as mean ± standard deviation. All pairwise multiple comparisons were calculated using non-parametric tests (Kruskal-Wallis and Conover-Iman combined tests and Mann-Whitney test) with $p<0.05$ significance level, and correlation analysis of the abundance of the different ARGs was performed using Spearman's rank correlation coefficients in XLSTAT v. 2020 (Addinsoft, USA).

Calculation of α-diversity and β-diversity of microbial populations was performed using PAST v4.09. The PERMANOVA analysis was used to determine whether the parameters studied were significant in terms of absolute quantification of target genes, physicochemical performance, presence of pharmaceuticals and dominant OTUs using PASTv3. In addition, the phylogenetic tree of the microbial communities was constructed by hierarchical clustering using PAST v3.4 software (Hurtado-Martinez et al., 2021).

Principal component analysis was computed using R project v4.2.1 software and CoDaPack. RDA was conducted to analyze the association between archaeal, bacterial, and fungal dominant phylotypes and physicochemical traits using Canoco 4.5 for Windows software (Rodriguez-Sanchez et al., 2016).

4

4-RESULTS

CHAPTER-I

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Abstract

Hospital wastewater contains several contaminants of emerging concern that cannot be removed by conventional treatment processes. Many of these emerging contaminants are pharmaceutical active compounds, which are found in hospital wastewater at high loads. The presence of these toxics affects to the performance of biological processes in receiving wastewater treatment plants. This research evaluated the capability of the aerobic granular sludge technology to remove pharmaceutical active compounds from hospital wastewater in a single chamber, which to date has not been investigated with real hospital wastewater. Despite the high non-biodegradable organic matter content, COD and BOD_5 removal reached 75 % and 100 %, respectively. Nitrogen removal ranged from 70 % - 90 %, and phosphate removal was maximum 50 %. The technology was able to efficiently remove antibiotics, antiepileptic and antidepressant drugs, whereas non-steroidal anti-inflammatory drugs were removed and released under oscillating patterns. The granular biomass increased in size, but it reduced the settling velocity. Bacterial and fungal communities were acclimated to pharmaceutical inlets, whereas the archaeal population had a progressive adaptation over time. The aerobic granular sludge technology is therefore a viable approach to enhance the disposal of real hospital wastewater prior to discharge into the urban wastewater network

Keywords: aerobic granular sludge, emerging contaminants, hospital wastewater, microbial dynamics, pharmaceutical active compounds

CHAPTER I: Treatment of hospital wastewater using aerobic granular sludge technology: Removal performance and microbial dynamics

1. INTRODUCTION

Hospital wastewater (WW) is a growing concern because it is constituted by toxic compounds such as pharmaceutical active compounds (PhACs), heavy metals, and infectious organisms that, when released into the environment, pose serious environmental and human health risk (Szabová et al., 2020). Hospital WW also contains high concentrations of ammonia, total nitrogen, total suspended solids and organic matter with a low biodegradability based on COD/BOD_5 ratio. Hospital WW is characterized by the presence of hazardous materials for the prevention and diagnosis of diseases (Olicón-Hernández et al., 2021), such as antibiotics, antidepressants, analgesics, antiepileptics, antineoplastics, estrogens, radionuclides, metals, anaesthetics, lipid regulators, antipyretics and solvents (Kumari et al., 2020). Usually, hospital WW discharges into the urban WW networks and both are co-treated in wastewater treatment plants (WWTPs), which could disturb the balance of physic-chemical and microbial performance, due to the intrinsic toxicity of hospital WW is 10 to 100 times higher than urban WW (Gonder et al., 2021; Tian et al., 2023). The conventional processes for urban WW are not specially designed for removing pharmaceuticals, metals, or pathogens. Thus, hazardous material would be released into the environment through WW treatment discharges when hospital WW are co-treated with urban WW in municipal WWTPs (Zhang et al., 2020d).

Some legal regulations have been established to define the management and treatment of hospital effluent before its disposal (Verlicchi et al., 2015), because reducing the toxicity of hospital WW is an optimal consideration first to discharge to the urban sewage to meet the revised directive concerning to emerging contaminants in urban WW (2022).

A deeper knowledge of the conventional parameters, chemical characterisation, microbiological load, and seasonal variation would allow the adoption and selection of the technical treatment train effective concerning raw water (Verlicchi et al., 2015; Gonder et al., 2021). In this way, technologies for hospital WW treatment have been gaining attention (Khan et al., 2022). Alternatives to conventional activated sludge (CAS) such as photocatalytic oxidation, electrochemical oxidation, advanced oxidation, and

membrane technologies are suitable for achieving high-quality effluents (Szabová et al., 2020; Pariente et al., 2022). Advanced oxidations are the leading studied technologies to reduce the PhACs in the hospital WW, for instance, ozonization can remove > 90 % of PhSCs (Antoniou et al., 2013; Szabová et al., 2020). Fenton and photo-Fenton systems, which could be applied to polishing post-treatment, reached to remove 90 % but this technology is scarcely explored for real hospital WW (Pariente et al., 2022). Only a few studies employed photocatalytic systems for treating real hospital WW, but Konstas et al. (2019) pointed out removal ratios close to 80 % for longer exposition (simulating summer), and in the range of 42-57 % for shorter exposition (simulating winter). Other technologies such as membrane bioreactors are suitable alternatives for treating hospital WW with PhACs removal values higher than 80 %, but some of the most recalcitrant compounds remain in the effluent and the clogging of pores reduces the efficiency (Chiarello et al., 2016; Khan et al., 2020b). In most cases, these technologies are expensive, with high energy and cost requirements, which are not feasible to implement in the hospital vicinity prior to discharge into the urban WW network (Nivedhita et al., 2022), therefore it is difficult to achieve successful implementation (Svebrant et al., 2021).

Aerobic granular sludge (AGS) technology is postulated as a technology that enhances the robustness of a biological system for treating pollutants in comparison with CAS due to its overwhelming benefits (Han et al., 2022). AGS technology solves a few of these aspects with respect to the advanced oxidations, such as the reduction of 20 % in space requirements compared with the CAS, a 20 % - 25 % reduction in operation costs and 24 % - 40 % lower electricity requirements (Gonzalez-Martinez et al., 2017). This technology is based on large spheres of microorganisms embedded in a tridimensional matrix, which allows the co-existence of aerobic, anoxic and anaerobic niches and, consequently enhances the simultaneous removal of nitrogen, phosphorus and organic matter (Muñoz-Palazon et al., 2020a,b; Nivedhita et al., 2022). The high biomass retention and the long sludge retention time promote the growth of specialist microorganisms with metabolic pathways for the degradation of toxic compounds in raw WW (Nancharaiah and Sarvajith, 2019; Rosa-Masegosa et al., 2022; Munoz-Palazon et al., 2023). Also, the strong biofilm conformed in the tridimensional matrix promotes resistance against the stress of toxic compounds, because toxicity does not affect the

single cells, providing an attractive advantage facing changes in loads and the diversity of pollutants (Shi et al., 2021; Muñoz-Palazon et al., 2022 a,b).

Here, we comprehensively profile the physicochemical performance of the AGS technology for the treatment of real hospital WW over a period of 150 days. We analysed the capability of the system to degrade pharmaceuticals and common pollutants (COD, N and P), describing the granular dynamics from a physical and microbial points of view. Overall, this study provides an economically viable and environmentally sustainable solution in one step, which could be implemented in the hospital vicinity before its disposal into the urban WW network to reduce the effects on urban WWTPs and avoiding damage to humans, animals and ecosystems.

2. MATERIAL AND METHODS

2.1 Start-up

An AGS system in a sequencing batch reactor (SBR) was operated in a cylindrical column with a height of 90 cm, an inner diameter of 7 cm and an operational volume of 3 L. The air diffusor was at the bottom of the reactor, with an airflow of 4.0 L min^{-1} using fine bubbles. The pH, temperature and oxygen probes were located at 0.35 m from the bottom. The pH was in the range of 7.6 to 7.9, temperature was 16 ± 0.7°C and dissolved oxygen was 9.1 ± 0.4 $mg\ O_2\ L^{-1}$, keeping close to saturation. The effluent output exchanged 50 % of the water volume. The batch cycles were as follows: filling with raw water (4 min), aeration (170 min), decanting stage (3 min) and effluent discarding (3 min). Each cycle lasted for 3 hours, and the hydraulic retention time (HRT) was 6 hours. The feeding of the reactor was performed with a peristaltic pump from the top (Watson Marlow, United Kingdom). The influent was raw water from the sewerage network of pipes from a hospital in Granada (Spain), collected once a week during the same temporal window (every Wednesday from 8:00 to 9:00 a.m.). For the inoculation of the bioreactor, 1 L of granules cultivated were used, which were fed with synthetic WW simulating urban WW following the solution described by Rosa-Masegosa et al. (2023). Granules were operated in the lab-scale reactor with synthetic WW until achieving high-performance removal for COD, BOD_5, N and P with values of 90 %, 94 %, 85 %, and 60 %, at 22 days. For 14 days the reactor was operated to ensure the stability of the biomass before feeding it with real hospital WW.

2.2 Physicochemical analysis

The granular sludge was analysed through mean size and settling time. The mean granular size was measured using a scalimeter with a representative number of samples (n = 35 ± 5 pieces) taken during the aeration period (Muñoz-Palazon et al., 2018). The settling velocity of the granules was measured using a 50 cm glass column with a manual chronometer, following the protocol described by Hurtado-Martinez et al. (2021). Biomass concentration (Mixed liquor suspended solids-MLSS) measurements in the bioreactor were performed according to the Standard Methods for the Examination of Water and WW (APA, 2012). COD and BOD_5 were determined in duplicate in the influent and effluent (APA, 2012). Nitrogen and phosphorous ion concentrations (NH^{4+}, NO^{3-}, NO^{2-},PO_3^{4-}) were analysed by ion chromatography (Metrohm Ion Chromatograph, AG, Switzerland) (Rodriguez-Sanchez et al., 2016).

2.3 Pharmaceutical compound extraction and quantification

Concentrations of PhACs were determined in triplicate (Baena-Nogueras et al., 2016). Briefly, solid phase extraction (SPE) was employed as the pre-treatment method (HLB cartridges Oasis, 200 mg). Conditioning of the HLB cartridges was performed using 8 mL of methanol and 8 mL of Milli-Q water. Subsequently, the samples (100 mL) were passed through the cartridges, and the cartridges were washed with Milli-Q water (10 mL) and dried in air. The final extract elution was evaporated under nitrogen stream and reconstituted with 2 mL of methanol-water in v/v 10:90 (Rueda-Márquez et al., 2021). Samples were kept at -20 ˚C until their processed within 2 months after.

The chromatographic separation was done with ultra-high performance liquid chromatography equipment (UHPLC 1260 Infinity II, Agilent), equipped with a Zorbax Eclipse plus C18 Column (3×50 mm-1.8 µm particle size; Agilent) under positive electrospray ionization for all compounds, except for diclofenac (DCL), naproxen (NXP) and triclosan (TRC) that was under negative ionization (Gallardo-Altamirano et al., 2021). The UHPLC was coupled in tandem with a triple quadrupole-QqQ 6470 LC/TQ (Agilent) with a quaternary UHPLC pump using gas flow of 0.4 mL^{min-1} at 350 ˚C. Data acquisition, qualitative analysis, quantitative analysis, mass spectral library management, and reports were calculated using MassHunter software (Agilent).

In previous studies, pharmaceuticals were selected due to their high-risk quotient, correlation to the study area or ecological risk (Khan et al., 2020b; Olicon-Hernandez et

al., 2021; Muñoz-Palazon et al., 2021b). For this study, seven drugs belonging to different therapeutic classes were selected based on their high risks: Antibiotic: trimethoprim (TMP); antidepressant: carbamazepine (CMP); antitumoral compounds: cyclophosphamide (CCLP) and TRC; anti-inflammatory compounds: ketoprofen (KTP), DCL and NPX (Olicon-Hernandez et al., 2021; Muñoz-Palazon et al., 2022a). Chemical solutions were also prepared as standard curves supplemented with 0.1% EDTA using different concentrations range of TMP (0.05-15 $\mu g\, mL^{-1}$), CMP (0.05-50 $\mu g\, mL^{-1}$), CCLP (0.05-15 $\mu g\, mL^{-1}$), TRC (0.05-15 $\mu g\, mL^{-1}$), KTP (0.5-500 $\mu g\, mL^{-1}$) DLC (1-200 $\mu g\, mL^{-1}$) and NPX (3-500 $\mu g\, mL^{-1}$), which covered the concentrations measured in 10 random samples from the raw HWW and treated water measurements prior to proceed with analysis.

2.4 Nucleic acid extraction and massive parallel sequencing

The granular biomass samples were collected, once the reactor was stable, at days 0, 3, 7, 15, 30, 60, 90, 120, 140 and 150. Representative granule samples were taken, submerged in a saline solution (0.9 % NaCl) and centrifuged at 5000 rpm for 20 min at 4 ˚C. Subsequently, the supernatants were discarded, and the pellets were stored at -20˚C. The nucleic acids were extracted in duplicate using the FastDNA SPIN Kit for Soil (MP Biomedicals, USA).

Next-generation sequencing (NGS) was carried out using the platform MiSeq Illumina of the duplicate DNA extracted. The duplicated DNA samples were amplified using the primer pairs: Bacteria807F (5′-GGATTAGATACCCBRGTAGTC-3′) and Bacteria1050R (5′-TAGYTGDCGACRRCCRTGCA-3′) for the amplification of V5–V6 hypervariable regions of 16S rRNA of *Bacteria*, ITS1S (5’-CTTGGTCATTTAGAGGAAGTAA-3’) and ITS2S (5’-GCTGCGTTCTTCATCGATG-3’) for *Fungi*. For the amplification of the 16S rRNA of *Archaea*, the primers 519SGF- (5’-CAGCMGCCGCGGTAA-3’) and 907SGR (5’-CCCCGTCAATTCCTTTGAGTTT-3’) were used (Muñoz-Palazon et al., 2022a).

2.5 Bioinformatic data curation

The next-generation sequences were subjected to a quality trimming analysis to keep the high-quality reads, using the open-access software MOTHUR (Schloss et al., 2009). For that, forward and reverse contigs were assembled with quality scores of a mismatched

base equal to 0. Then, reads with more than eight homopolymers or any ambiguous base were removed, and the remaining reads of *Archaea* and *Bacteria* that did not align properly on the forward and reverse positions against the SILVA seed database were eliminated using the Needleman conditions. For all targets, chimerical reads were detected and removed using the VSEARCH algorithm, and reads that belonged to target genes different from the focus were discarded (Muñoz-Palazon et al., 2022b). The clusters of sequences were calculated using the abundance-based greedy method with a similarity of 97 % for *Bacteria* and 95 % for *Fungi* and *Archaea*. The operational taxonomic units (OTUs) results of taxonomic consensus were affiliated with SILVA_nr data for *Archaea* and *Bacteria* and UNITE for *Fungi*. Finally, singleton OTUs were eliminated to build the OTU tables. For the construction of community maps, it was used the average abundance of duplicates for each sample day and target gene.

2.6 Real-time PCR

Real-time PCR (qPCR) assays were performed using extracted DNA from biological samples in Quant Studio™ 3 Real-Time PCR Systems. *Bacteria*, *Archaea* and *Fungi* were quantified using diagnostic 16S rRNA genes and 18S rRNA with primers 341 F-534 R (Muyzer et al., 1993), ARCH915R-UNI-b-revF (Yu et al., 2008) and FungiQuantF-FungiQuantR (Liu et al., 2012). Genes involved in ammonia oxidation and nitrate denitrification were targeted with *amoA* and *nosZ* genes, respectively (Henry et al., 2006; Rotthauwe and Witzel, 1997). For phosphate-accumulating organisms, 16S rRNA of *Candidatus Accumulibacter* was used as a proxy for polyphosphate-accumulating organisms (PAO) (Correa-Galeote et al., 2021).

The reaction mixture of 25 µL was composed as follows: 19.36 µL DEPC sterile water, 2.5 µL buffer with $MgCl_2$, 0.5 µL dNTPs (8 mM), 0.15 µL of each primer, 0.125 µL Taq polymerase, 0.125 µL SYBR Green (x20) and 0.0625 µL BSA (BioLabs) (Muñoz-Palazon et al., 2020b).The primer pairs and annealing conditions are described by Muñoz-Palazon et al., 2019 and Correa-Galeote et al., 2021. Raw data were processed using the proprietary QS3 software (Applied Biosystems, Thermo Fisher Scientific). Gene quantification was normalized both by biomass and the volume of nucleic acids.

2.7 Statistical analysis of diversity, similarity and multivariate

The α-diversity of archaeal, bacterial and fungal populations was calculated using the software PAST v. 4.09, applying the OTU table considering the indices of Chao-1,

Shannon-Wiener, Simpson, Pielou's evenness and Berger-Parker, which were calculated with a 97% confidence range by 999 bootstrap replications. The Whittaker index was performed to capture the β-diversity of all phylotypes among pairs of samples, using the OTU table in the PAST v. 4.09 software.

A PERMANOVA was performed to evaluate if the studied parameters were significantly related to the absolute quantification of genes, the physicochemical performance, the presence of pharmaceuticals and the dominant OTUs. The PERMANOVA was computed using PAST v. 3 with Bray-Curtis distance and under 9.999 permutations. The reads of the OTU table sequence samples were used for the calculation of the trees of *Archaea*, *Bacteria* and *Fungi* communities throughout the operation period. The phylogenetic tree was built by hierarchical clustering, using under 9.999 bootstrap replications following the Bray-Curtis model and using the PAST software v. 3.4. (Hurtado-Martinez et al., 2021).

Principal components analysis (PCA) was calculated using the R project v. 4.2.1 software and CoDaPack. Raw data were pre-treated as follows: a) correction to avoid zero values, b) transformation to the centred logarithm and clustering the compositional data of the OTU table of the microbial dynamics by distance distribution. To complement these results, a SIMPER analysis was done to select the phylotypes that contributed to dissimilarities between samples before and after steady-state (supported by PERMANOVA analysis), using the PAST software with dissimilarities with the Bray–Curtis distance.

Linkages among archaeal, bacterial and fungal dominant phylotypes and physicochemical characterisation were analysed using multivariate redundancy analysis (RDA). Linkage was determined using the centred logarithm of each parameter computed using 499 unconstrained Monte-Carlo simulations under a full permutation model, applying Canoco 4.5 for Windows (Rodriguez-Sanchez et al., 2016).

3. RESULTS AND DISCUSSION

3.1 Effects of the treatment of hospital wastes in sewage on the performance of AGS

This experiment was carried out for 150 days, and the obtained results of the physicochemical analyses highlighted two removal phases (Figure 1). The existence of two different phases was corroborated by one-way PERMANOVA (Table S1). Between

these two stages, nitrogen, phosphate, COD and BOD_5 removal and the settling velocity of granules were statistically significant ($p < 0.05$), whereas mean granule size was not statistically significant ($p > 0.05$). Based on these results, the experiment was studied into two phases: start-up and acclimatization (until day 37) and steady-state (from day 38 to day 150).

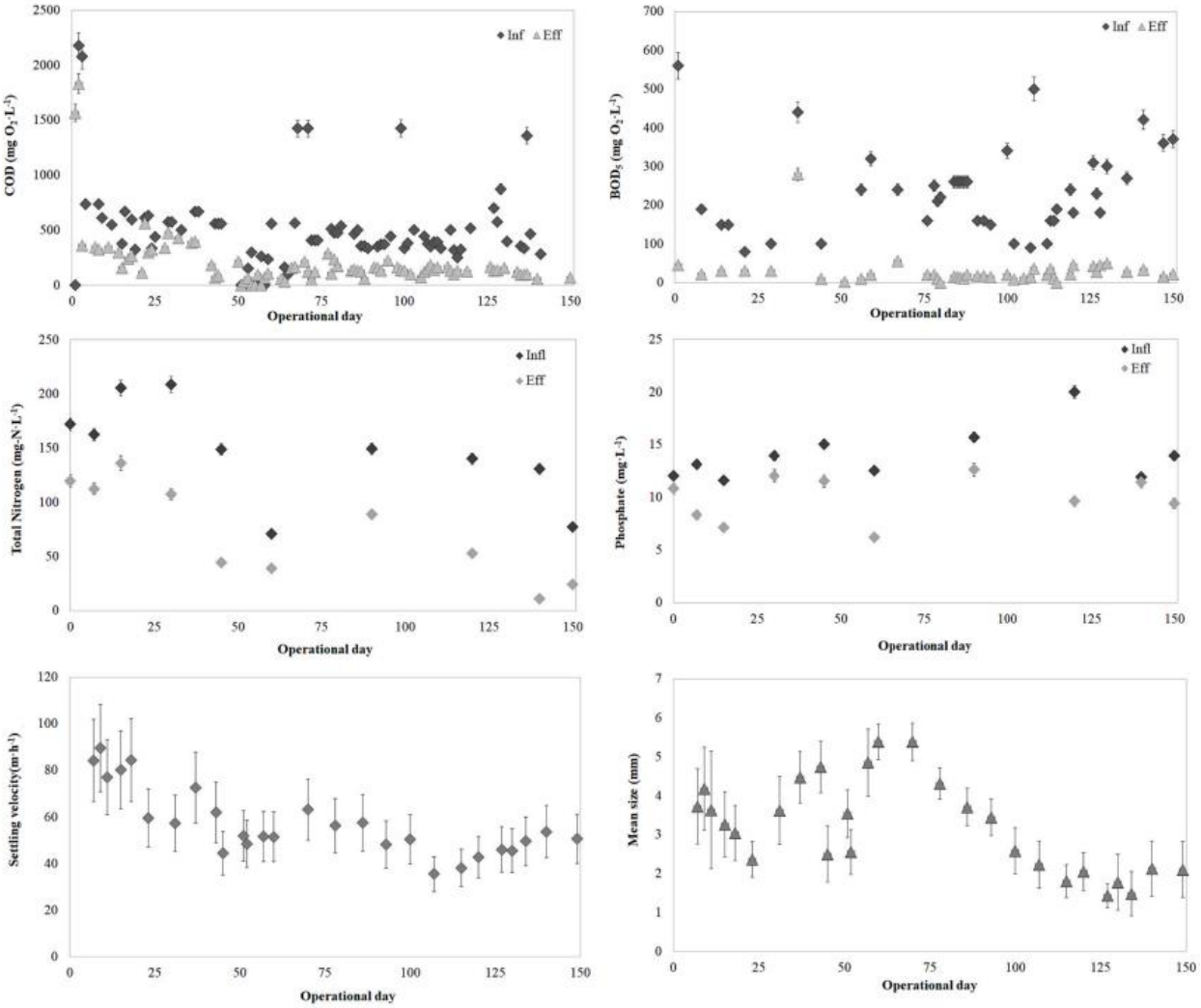

Figure 1. Pollutant removal and AGS characterization: COD (a); BOD_5 (b); total nitrogen (c); phosphate (d) in mg L^{-1} in influent and effluent; mean size (e) and settling velocity (f) of granular biomass.

The hospital WW characteristics were highly variable over operational time, as it is reported in other studies carried out with real hospital WW (Khan et al., 2020b; Gonder et al., 2021; Bijlsma et al., 2021). Organic matter, measured by BOD_5 and COD, was monitored throughout the operation period. The COD values oscillated, with an average value of 514 mg O_2 L^{-1} and values of standard deviation of 391.83 mg O_2 L^{-1} , caused by the COD peak concentration of up to 2100 mg O_2 L^{-1}, but these trends are common

values in the hospital WW inlet (Khan et al., 2020b). During the start-up phase, the COD removal ratio showed no obvious pattern, and the variability in the removal percentage was not strictly linked with the COD concentration in the influent. These results could be caused by the acclimatization process to the nature WW with a high load of pollutants and microbial loads, as well as by the biodegradability of WW. From day 38, COD removal ratios were higher than 50 %, but during most operation the ratio was higher than 75 %. The COD removal ratio was strongly linked with the content of non-biodegradable matter, which corroborated with the mean COD/BOD_5 ratio of 2.77 ± 1.70, demonstrating the low degradability of hospital WW as reported Bhandari et al., 2023. Figure 1 showed the BOD_5 (biodegradable organic matter) influent concentration, which was in the range of 100 to 300 mg O_2 L^{-1}; the effluent concentration in the steady-state was lower than 25 mg O_2 L^{-1} throughout the experimental period, achieving a removal rate close to 100 % (Supplementary Material).

The nitrogen influent values were in the range of 130 to 170 mg N L^{-1}, but values higher than 200 and lower than 70 mg N L^{-1} were also detected (Figure 1). The removal ratio was close to 70 %, although it was imperative to note that ammonium oxidation was close to 90 % (Figure S1). The mean phosphate concentration in the influent was 13.74 ± 2.69 mg $PO_3^{4-}L^{-1}$, whereas that in the effluent ranged from 12 to 6 mg L^{-1}. The removal ratios were not competitive during the start-up phase, and in the steady state achieved maximum performance of 50 % (Figure S1), obtaining higher efficiency under low N:P ratio. The N:P ratio largely oscillated throughout the experimental period, with values in the range of 3.52 to 17.70 and an average of 11.04 ± 4.44.

Regarding the COD:N ratio, the best performance was obtained with values close to 3.6:1, resulting in a COD removal close to 80 % and nitrogen removal in the range of 62 % to 70 %. This COD: N ratio was lower than that described by Hamza et al. (2022), who used synthetic WW, but the performance met the requirements of the European legislation, even at high pharmaceutical and microbial loads. Nivedhita et al. (2022a) showed in their research as a single antibiotic had a negative impact on the removal performance in comparison with a control bioreactor operated without PhACs. To date, this is the first research that treats real hospital WW with AGS for testing the technology, and considering the obtained results, AGS system could reduce substantially nutrients load in presence of toxic compounds from hospital WW.

From the physical point of view of granules, hospital WW had impact on the mean size and settling velocity (Figure 1). The mean granular size reached the maximum after the stabilisation (day 55). Nevertheless, from day 75 onward, granular size progressively decreased with diameter close to 2 mm, slightly larger than the granules described by Nivedhita et al. (2022a) in the presence of oxytetracycline. In this case, smaller sizes of granules had advantages such as an increase in the specific surface area in contact with the pollutants, and the granules were more compact and smoother than those used as inoculum. Granule color changed from yellow to brown, probably because of the organoleptic characteristics of the real hospital WW, because previously granules were cultivated with synthetic WW. It is important to mention that despite the changes in mean size, the PERMANOVA results showed no statistically significant effect on the two phases ($p > 0.05$). The settling velocity decreased progressively from start-up until day 40, whose values remained in the range of 40 to 60 m h^{-1}.

It has been suggested that PhACs and personal care products (PPCPs) could improve the settling capacity of granules (Muñoz-Palazon et al., 2022a; Česen et al., 2015). However, in this study, the aerobic treatment of real hospital WW did not enhance the settling performance of the granular sludge, and the results observed in previous studies could be explained by the controlled conditions (Muñoz-Palazon et al., 2022a; Česen et al., 2015).

3.2 Occurrence of pharmaceutical compounds in urban wastewater and after AGS treatment

Seven PhACs were analysed in influent and effluent samples. The values were significantly higher than those observed for influent and effluent samples of urban WWTPs due to the hospital origin (Figure 2). CCLP is a widely used drug in breast cancer therapy and one of the oldest and most widely prescribed alkylating cytostatic medicine (Česen et al., 2015). It has previously been observed at a concentration range of 0.014-22 μg L^{-1} in European hospital WW. In this study, the concentration of CCLP was evenlower ranging from 0.02 to 0.25 μg L^{-1} in the raw WW, and the removal rate was 100% for the entire experimental period (Figure. S2).

TRC has been frequently detected in aquatic ecosystems and potentially damages various organisms; its removal has therefore attracted considerable interest. In municipal and hospital WW, TRC has been detected in the range of 0.07-14,000 μg L^{-1} (Luo et al., 2019). In this study the TRC concentration in hospital WW was <0.2 μg L^{-1} throughout

the experimental period. However, at day 15, the removal performance was negative; it could be caused by bioaccumulation or release processes, whereas the average removal ratio was 60 %. These rates are comparable with those reported before, ranging from 35 % to 69 % (Archana et al., 2017; Luo et al., 2019; Mohan and Balakrishnan, 2019). The variations in the TRC concentration in the hospital WW discharged can be linked to seasonal changes as TRC is largely used in personal care and biocidal products (Mohan and Balakrishnan, 2019) .

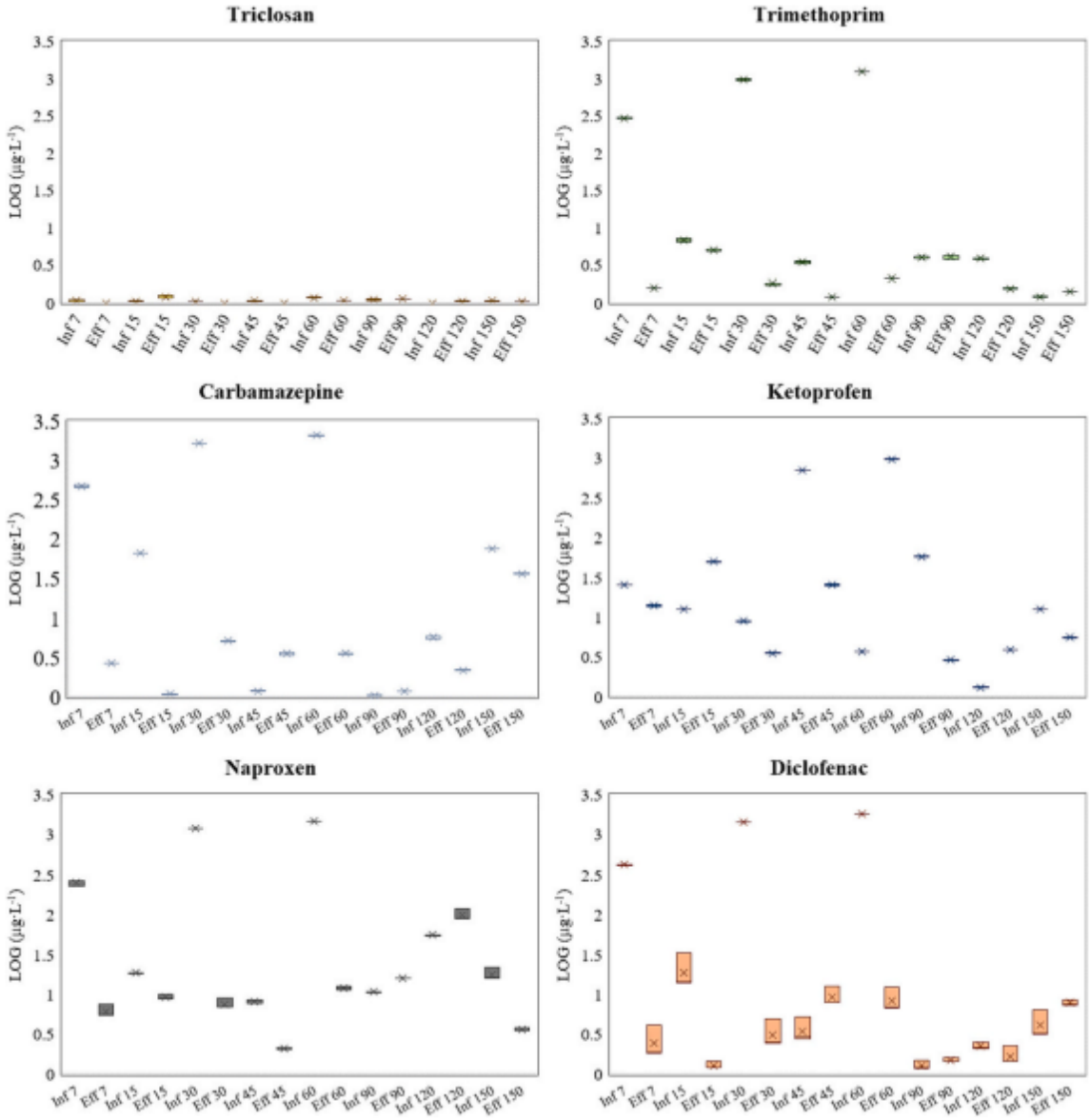

Figure 2. Performance of treating the influent and effluent of hospital wastewater measured in triplicate in key days.

TMP is one of the most used antibiotics worldwide, and its concentration in urban WW depends on several factors. In this study, the TMP variation varied from 0.21 to 1240

µg L^{-1} in the HWW discharged, but there was no seasonal pattern. The obtained results indicated that TMP was efficiently removed using the AGS technology, with a removal rate from 80 % to 99 %, but the results suggested possible release processes at days 90 and 150. These results pointed out the ability of AGS to remove high loads of this pollutant, such as at days 30 and 60, when the concentrations in the influent and effluent were 960-1240 µg $L^{-1}$and 0.74-1.11 µg L^{-1}, respectively. The values of this matrix were higher than those usually reported, such as 6.6 -7.7 µg L^{-1} (Lien et al., 2016; Ngigi et al., 2020). Moreover, the AGS technology has previously been described as a potential technology for TMP degradation (Muñoz-Palazon et al., 2021b, 2022b). The reactor degraded 50 % of the CBP during the experimental period and in almost 100% on the 1/4 of the experimental time. The CPB removal ranged from 52 % - 74 %, although on days 45 and 90, the removal was negative. During these days, the CBP concentration of the influence was lowest (<1 µg L^{-1}), and the concentration in the effluent did not exceed 2.5 µg L^{-1}. These results demonstrate the ability of AGS to significantly reduce the high loads emitted by hospital WW.

The NPX removal had oscillated values because of influent concentration and possible removal-release processes. At the beginning, the removal rate ranged from 44 % - 99 %, whereas the system released more NPX on days 90 and 120, when the granules were long time exposed to PhACs, previously reported by Bessa et al. (2022). The same pattern followed the DCL discharged by the hospital, revealing oscillating concentrations from 0.29 to 1800 µg L^{-1} over the experimental period. The removal performance was highly efficient under high influent loads, but the positive-negative rate of DCL removal could be linked to the desorption process as reported Bessa et al. (2022).

KTP was the most recalcitrant compound, although the system was able to remove average values of 50 % of the KTP concentration following the results reported Arcanjo et al. (2022). In fact, the results of this research showed a negative removal rate of -320%, could be caused by the accumulation over long time as pointed out Arcanjo et al. (2022). It is well known KTP can adsorb on suspended solids and sludge during WW treatment, and it has the highest tendency to attach onto particles among the studied compounds such as CMP or DCL as corroborated by many authors (Lindholm-Lehto et al., 2015; 2018). Revisions about the issues derived from KTP highlighted that full-model of the adsorption of this compound is still lacking, the best option to remove KTP from the aqueous phase is absorption processes (Georgin et al., 2022).

Based on these results, the surface of granular sludge could promote the bioadsorption-desorption processes, especially in terms of nonsteroidal anti-inflammatory drugs (NSAIDs) such as NPX, DCL and KTP (Lindholm-Lehto et al., 2015, 2018; Olicon-Hernandez et al., 2021; Muñoz-Palazon et al., 2022b). AGS could be postulated as a technology able to remove more efficiently PhACs than CAS in a single stage, which could be implemented as pre-treated to reduce the negative impact of the toxicity prior to disposal in the UWW networks (Rueda-Márquez et al., 2021).

3.3 Absolute quantification of microbial population dynamics

The absolute quantification of target genes showed a trend led by the drug concentration entering the system (Figure 3). The 16S rRNA levels of *Bacteria* and *Archaea* were in the range of 10^{10} to 10^{12} and 10^{7} to 10^{8} magnitude orders, respectively, until day 60. The highest loads of TMP, CPB, NPX and DCL were detected on day 60; subsequently, the numbers of bacterial and archaeal 16S rRNA genes abruptly decreased by 2-3 magnitude orders, followed by a later recovery prior to the shock. These results suggest that the high loads affected the bacterial and archaeal populations, but the robustness of the microbial population in the granules allowed to retrieve the damage caused by the PhACs compounds due to the different mechanisms of resilience in the microbial community in a temporal line (Philippot et al., 2021). This effect was even stronger in communities of fungal and phosphate-accumulating organisms (PAO), with a total disappearance at day 120. It is a novel picture of the most prolonged effect in time that has the high load of drugs in the PAO and fungal communities, as reported Huang et al. (2021), which follows the trajectories of complete recovery because of ecosystem succession that promote return to the original state (Philippot et al., 2021).

Granules are comparable to biofilms with several niches, and generally, PAO and fungal communities are contained in the interlayer zones and the core, respectively (Nancharaiah et al., 2019; Muñoz-Palazon et al., 2020a). The spatial situation of PAO in the anoxic zone could exert a buffer effect in these communities since many authors describe how AGS systems have advantages since the toxic compounds do not reach individual cells but are distributed in all consecutive cells (Nancharaiah, Y. V., Kiran Kumar Reddy, 2018; Correa-Galeote et al., 2021). In the present study, the deeper localisation of these microorganisms facilitated their presence until day 90. However, the PAO suffered in the later stages, when the PhACs had passed through the external layers of the granules to the interlayer, because the PAO organisms are sensible when they are directly exposed to

PhACs (Huang et al., 2021; Nivedhita et al., 2022). The fungal population has been described as an essential part of the microbial community in biological WW treatment, with the ability to transform and degrade PhACs, but sometimes the number of fungal copies are below of detection limit using a specific primer set for Fungi (Cruz-Morató et al., 2014; Maza-Márquez et al., 2019; Gallardo-Altamirano et al., 2021). The number of gene copies of PAO and fungi increased at day 150 to values similar to those before the shock. Although denitrifiers, analysed by the *nosZ* gene, were also affected over long exposition, the copy number was only slightly affected. The *amoA* gene was not negatively impacted by the drug load in the influent, in contrast to the remaining functional genes. The number of copies trended to increase progressively over time, albeit with large fluctuations.

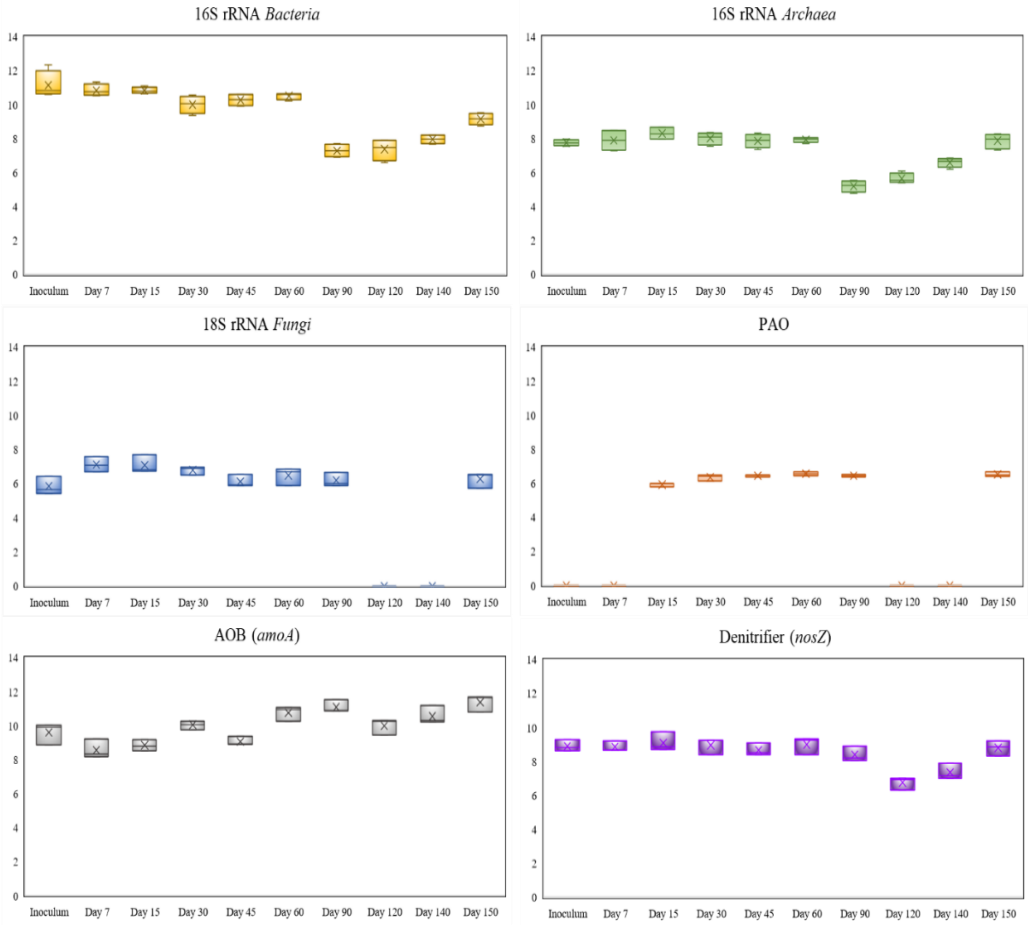

Figure 3. Gene copy numbers (Log No. of copies/gram of granules) determined by quantitative PCR in granular sludge treating hospital wastewater.

3.4 Dynamics of the microbial population in granules

3.4.1 Diversity of the archaeal population

Archaea are microorganisms with great ability to colonize the majority of environments in nature, but they have been less studied due to the lack of knowledge about their metabolisms (Aparici-Carratal et al., 2023). In biological WW, they play an essential role in the degradation of pollutants such as nitrogen, phosphate, aromatic hydrocarbons, chlorinated compounds, sulfur compounds, and heavy metals, among others. The archaeal population in the granular sludge treating real hospital WW was more diverse compared to previous findings obtained via NGS (Muñoz-Palazon et al., 2018). Overall, 21 OTUs were represented by a relative abundance of >1.5 % (Figure 4).

In the granules employed as inoculum, the most dominant phylotypes were *Methanocaldococcus* and an unclassified *Methanococcales* order, both accounting for >20 % of the total inoculum. Three OTUs belonged to unclassified *Bathyarchaeota*, which played an important role in the inoculum. The archaeal community, with great diversity and evenness, changed slightly until day 15, with slight modifications related to the increase of an Otu01 belonging to *Bathyarchaeota* and Otu04 affiliated to *Methanospirillum*. However, a remarkable change in the population was observed via hierarchic clustering, which showed significative modifications before and after Day 30. From day 30 to the end of the experimental period, the role played by *Methanocaldococcus* was greatly reinforced, reaching 50 % of the total relative abundance.

Following the trend observed for the qPCR results, at day 90, the community was dominated by three OTUs, two of which belonged to an unclassified *Bathyarchaeota* and *Methanocaldoccus*, possibly caused by the high drug loads in hospital WW. The proliferation of OTUs belonging to *Thermoprotei*, *Methanospirillum*, *Methanococcales* and *Halobacteriaceae* increased the diversity of phylotypes in the population dynamics. These results are interesting because the common perspective excludes the domain *Archaea* from biotechnological approaches except in anaerobic digestion, but these findings show that archaeon could play an unknown role in the removal of drugs from HWW. Few authors have reported the synergetic effects among *Archaea*, *Bacteria* and *Fungi* in the degradation of refractory organics (Fan et al., 2021). Although few studies have incorporated the massive parallel sequencing of the archaeal 16S rRNA gene, to our knowledge, the is the first time *Archaea* were studied in a granular sludge (biotechnological WW treatment approach) treating real hospital WW.

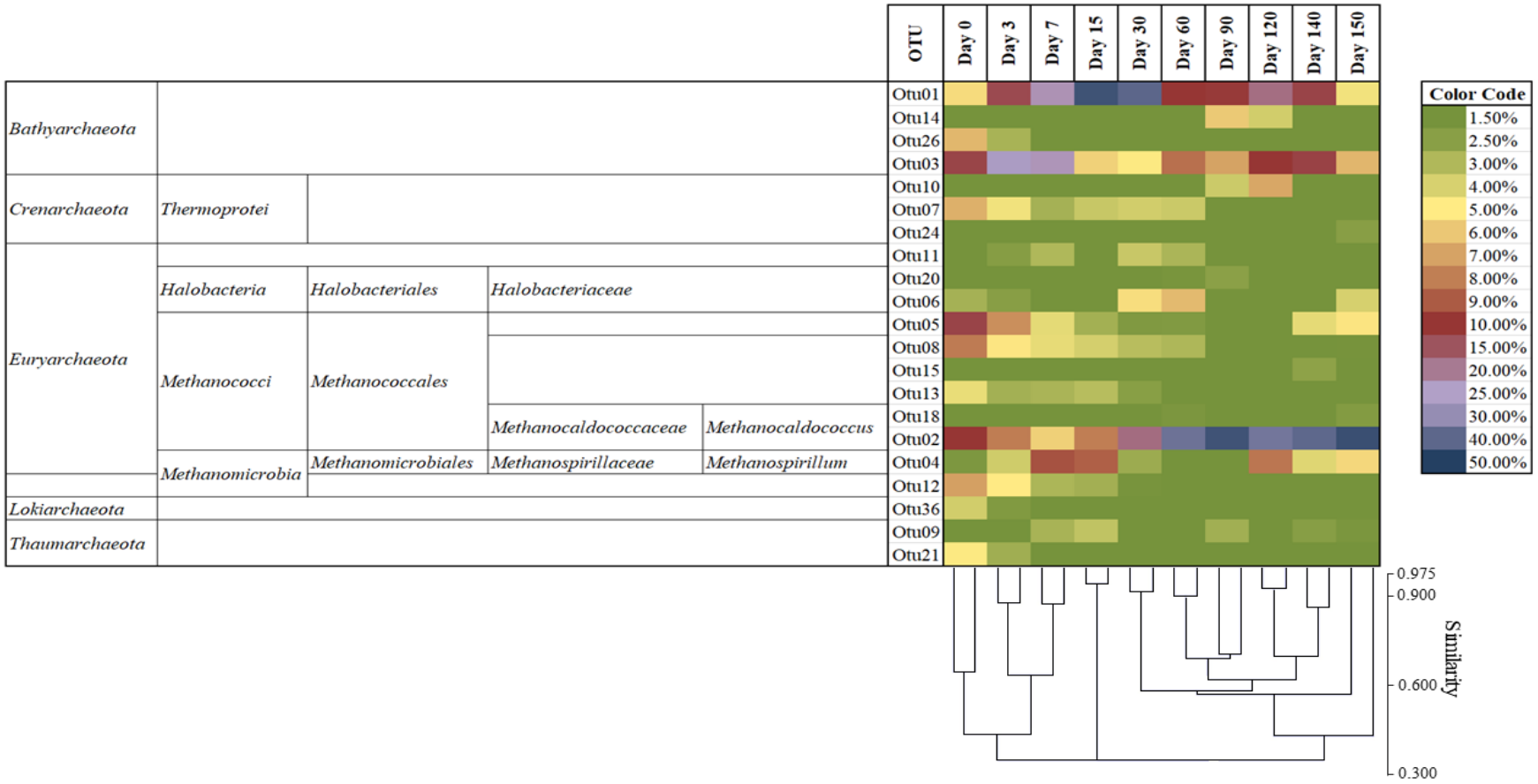

Figure 4. Dynamics of the archaeal population in aerobic granular sludge treating real hospital WW for long-term operation linked with hierarchical clustering.

3.4.2 Diversity of the bacterial population

The bacterial population dynamics revealed a great diversity of OTUs linked to processes depending on the raw WW. Figure 5 shows the relative abundances of bacterial genera regardless of the clustering calculated for the OTUs. Figure S3 shows the complete heat map with bacterial OTU clustering at 97 % similarity.

The population in the inoculum was represented by the *Dokdonella*, unclassified *Comamonadaceae* and *Hyphomicrobium*. *Dokdonella* is an aerobic denitrifying bacterial genus found in biofilms of AGS systems, even in the presence of pharmaceuticals, which had been described with a positive correlation with the existence of antibiotic-resistant genes (ARG) (Shi et al., 2021; Zou et al., 2023). *Hyphomicrobium* is a denitrifier with the ability to accumulate phosphate, according to Yuan et al. (2019). Moreover, this genus plays an important role in granule formation and compactness because it excretes extracellular polymer substances (EPS) under high aeration pressure (Sun et al., 2022). Nonetheless, the excessive proliferation of this genus could cause the destabilization of the granules. *Comamonadaceae* is a family widely described in AGS systems, suggesting its function in granule stability, provided by its high ability to produce EPS (Mery-Araya et al., 2019).

In the present study, from day 3, the granular community began to change, induced by the nature of hospital WW and its microbial load. Although *Dokdonella* and *Comamonadaceae* phylotypes were present, *Hyphomicrobium* abundance declined sharply, whereas several genera taxonomically affiliated with *Leadbetterella*, *Flavobacterium*, *Brevundimonas*, *Thauera* and *Rhodobacteraceae* unclassified proliferated. The influent composition encouraged the proliferation of several bacterial phylotypes competing for carbon and nutrient sources. This trend was observed until day 7, when *Leadbetterella* acquired >30 % of the total relative abundance. This genus has also been observed in AGS treating pharmaceutical WW, and its relative abundance increased with increasing loads (Jiang et al., 2021).

The changes mentioned in Section 3.2, given the real characteristics of hospital WW, modified abruptly the bacterial population because at day 15, any phylotype exceeded 5 %, whereas at day 30, new taxa started to play important roles, such as *Leucobacter*, *Xanthomonadaceae*, *Peptostreptococcaceae* and *Macellibacteroides*. In contrast, the phylotypes previously found in the system, such as *Comamonadaceae*, *Flavobacterium*,

Hyphomicrobium and *Rhodobacteraceae*, reached 40%, 18%, 15% and 12%, respectively, of the total relative abundance. The oscillation in the drug loads between days 15 and 30 could induce the changes in microbiome, highlighting the low drug loads at day 45, with a subsequent increase at day 60. The alternations resulted in vast modifications in the bacterial dynamics. The influent of day 60 showed the highest drug loads, with obvious effects on the microbiota, which was corroborated by the proliferation of 16 OTUs with relative abundances of >7.5 %, some of which have not previously been described in this biological system, such as *Mycobacterium*, *Demequinaceae*, *Microbacteriaceae*, *Dysgonomonas*, *Cytophagaceae* (Olicón-Hernandez et al., 2021), *Runella*, *Taibaiella* and *Fusibacter.* These phylotypes only were present at Day 60.

The changes in the bacterial population, demonstrated by means of mean size, settling velocity and removal performance, are interesting because the granules represent robust biofilms that can resist considerably changes in influent characteristics, despite changes in the microbiome. Hierarchical clustering revealed as day 60 act the frontier between the two stages for bacterial population. Possibly, the toxicity of the influent negatively impacted the bacterial community, which agrees with the decrease in the copy number of the 16S rRNA gene of *Bacteria* at day 90. Consequently, any phylotype exceeded the 2% of relative abundance, and only *Comamonadaceae* and *Dokdonella* showed abundances similar to those in the initial phase. Once the drug load remained stable, from day 120 to day 150, the microbial community denoted certain stability whose community was dominated by *Saprospiraceae*, *Comamonadaceae*, *Xanthomonadaceae* as well as the genera *Flavobacterium* and *Comamonas*. *Saprospiraceae* (*Bacteroidetes* class) preferentially degrades complex organic compounds as carbon sources (Wang et al., 2018c). Moreover, some interesting genera involved in nitrogen metabolism appeared during this period, such as *Diaphorobacter*, which simultaneously performs nitrification and denitrification (Khardenavis et al., 2007), or the ammonia-oxidising genera *Acinetobacter* and *Comamonas* (Ouyang et al., 2020; Zhu et al., 2023). These taxa represent the core of the bacterial population, which promotes granular stability because they were present in both the inoculum and after recalcitrant bioremediation. The use of inoculum, regardless of their proceed, could promote the proliferation of specific genera able to remove recalcitrant compounds, remaining stable in its hard population core (Muñoz-Palazon et al., 2020b). Finally, few potential human pathogens were present in

the granules, such as *Enterococcus*, *Mycobacterium*, *Dysgonomonas* and *Clostridiaceae*, among others, bacterial pathogens commonly found in hospital WW.

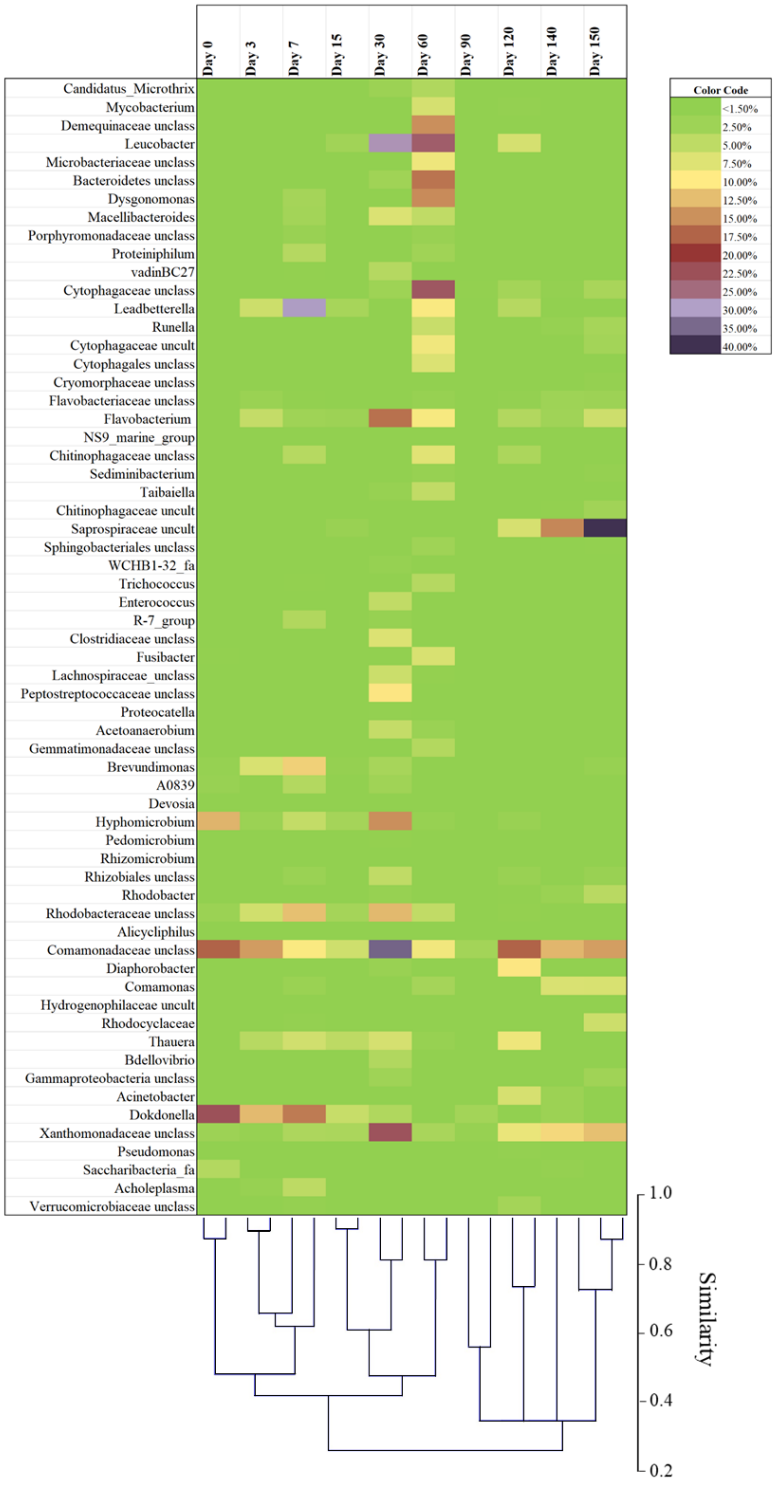

Figure 5. Heat Map of the dominant bacterial taxa in the granular sludge system treating hospital WW for long-term operation, linked with hierarchical clustering.

3.4.3 Diversity of the fungal population

Throughout the experimental period, there were 43 representative OTUs with a relative abundance of >1.5 % (Figure S4), but due to the huge fungal diversity were represented on Figure 6 by its taxonomic affiliation. *Nectriaceae* was the most abundant family in the inoculum, with a relative abundance above 70 %. In addition, the *Hypocreales* order and the *Herpotrichiellaceae* family were represented with relative abundances of 15 % and 9.5 %, respectively. *Nectriaceae* played an essential role in granular biomass; during the first operation stage, its relative abundance peaked, reaching 96 % on day 30. Some members of the family *Nectriaceae* are emerging and opportunistic human pathogens (Batista et al., 2020); some species of this family have been reported in hospital water systems, posing a significant infection risk (Kaur et al., 2020).

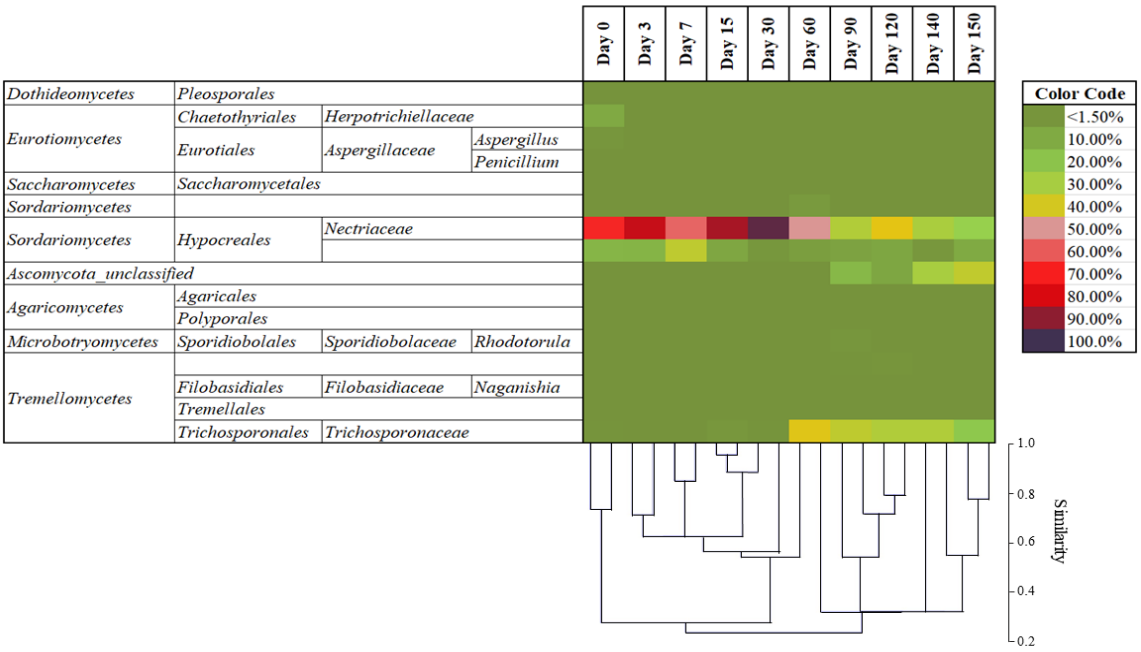

Figure 6. Heat map of fungal population dynamics in a granular sludge system treating WW for long-term operation linked with hierarchical clustering.

Similar to the archaeal and bacterial communities, the fungal population had a strong impact at day 60 because the abundance of the *Nectriaceae* family was decreased from 96 % to 46 %. At this point, the *Trichosporonaceae* family acquired a significant role in the community, along with the *Nectriaceae* family. Subsequently, a progressive decrease in the abundances of *Nectriaceae* and *Hypocreales* were observed, whereas that of *Ascomycota* decreased. In this way, the population in the granular sludge was not dominated by one specific phylotype, and the families *Nectriaceae* and

Trichosporonaceae as well as the phylum *Ascomycota* play functional roles in WW treatment (Maza-Marquez et al., 2016). The family *Trichosporonaceae* could act with syntrophic relationships with denitrifying bacteria, assisting them in the ring opening and chain breaking of organics (Fan et al., 2016). Some members of this family have been described as promoters of sludge bulking (Zhang et al., 2019a), but filamentous growth could be crucial in granulation, with filamentous microbes acting as the core of granules and a later bridge union with the ability to immobilize other microorganisms, as reported by Gonzalez-Martinez et al. (2018). In this study, they were ubiquitous during the entire experimental period.

3.5 Alpha- and beta-diversity analysis

The α-diversity indices for the archaeal population showed similar values from inoculum to the end of the experiment in terms of diversity and evenness, but the species richness, as indicated by the Chao index, increased over time from 60 ± 14 to 173 ± 3 (Table S2). In general terms, the species richness of bacterial communities increased over time, which was also observed for evenness. The diversity measured by Simpson indices showed values in the same range. The species richness of the fungal community significantly increased during the initial operation phase and remained stable during the 30 days. However, a specialization of granule-forming fungi was detected from day 90 to the end of the operation, based on the decrease in the Chao index. This was corroborated by the trend observed in the fungal community studies. The opposite trend was observed for diversity and evenness indices, which greatly decreased in the initial stage and started to increase from day 60 onward, exceeding the values of the inoculum. Based on these results, the bacterial and fungal communities play an important role in the formation of the granules and in the degradation of several PhACs, whereas the archaeal populations were not strongly modified.

The β-diversity, calculated using the Whittaker index, indicated an increase in the similarity between archaeal populations for pairs of samples throughout the experimental period, with higher dissimilarities between days 140 and 150 (Figure. S5). The dynamics of the bacterial communities revealed a progressive tendency to differentiate between pairs of samples, demonstrating the change produced at day 60, when the loading of PhACs was highest, with TMP >1000 μg L^{-1}, CMP >2000 μg L^{-1} NPX >1400 μg L^{-1} or DCL >1800 μg L^{-1}; similar findings have been reported previously (Zhao et al., 2015;

Vasiliadou et al., 2018). Although over time, the fungal communities showed values comparable to those of the bacterial populations, the trend was not as clear. In this case, the community at day 140 played a significant role in the dissimilarity with the remaining samples, with the highest values for all domains.

3.6 Clustering distribution and phylotype dissimilarity contribution analysis

The results of the PCA indicated significant differences among the granular archaeal communities over time, with three clusters of sample pairs (Figure S6a). The first cluster contains samples from start-up to day 15 were clustered, the second cluster contains samples from days 30 to 90, and the third cluster contains samples from days 120 to 150. The PCA demonstrates the logical sequential changes suffered by the archaeal population, as shown in the heat map of the *Archaea* domain. Moreover, the distance found among archaeal samples over time was caused by specific phylotypes calculated by SIMPER analysis (Figure S7). The taxa that contributed to this distance were *Methanocaldococcus* (33 %) and two OTUs belonging to *Bathyarchaeota* (27 %). This trend was observed in the archaeal population dynamics (Section 3.4.1), but both statistical analyses supported the hypothesis of the displacement of *Bathyarchaeota* by *Methanocaldococcus* in the steady-state period.

The PCA of the bacterial samples did not reveal any differences between the start-up and the steady-state stages, with a short distance between days 0 and 150 (Figure S6b). These findings can be linked to the high levels of diversity obtained in all samples, where the phylotypes had a high evenness. Based on the results of the SIMPER analysis, only 15 genera contributed to dissimilarities between both periods with >1.5 %, whereas communities with a lower diversity reported a major number of contributions in AGS systems (Rosa-Masegosa et al., 2023). The families *Saprospiraceae* and *Comamonadaceae* and the genera *Dokdonella* and *Leadbetterella* genera contributed >28% to the dissimilarities; these changes were clearly observed in the dynamics population studies (Section 3.4.2).

Finally, the PCA performed for the fungal community highlights the unclear and progressive distance between samples over time, but the clustering did not define the operational period, in contrast to *Archaea*. For days 3, 7 and 150, the distances were short, although the fungal phylotypes did not reveal high similarities. The phylotype that most contributed to the dissimilarities between the start-up and the steady-stable stages was

Nectriaceae (with a contribution of 40 %), which was the most abundant phylotype in terms of relative abundance in granules across the entire experimental period. However, its relative abundance greatly decreased over time. *Trichosporonaceae* proliferated with the maturation of the granular sludge system, but it was not present in the inoculum; this is in agreement with the findings of Gonzalez-Martinez et al. (2018). This phylotype contributed with 30 % to the dissimilarities between the start-up and steady-state periods.

3.7 Multivariate redundancy analysis

Multivariate redundancy analysis was performed to link the copy numbers of genes and the archaeal, bacterial and fungal communities with the performance evolution of the system. The linkage of absolute abundance genes analysed by qPCR and the physico-chemical parameters is shown in Figure 7.

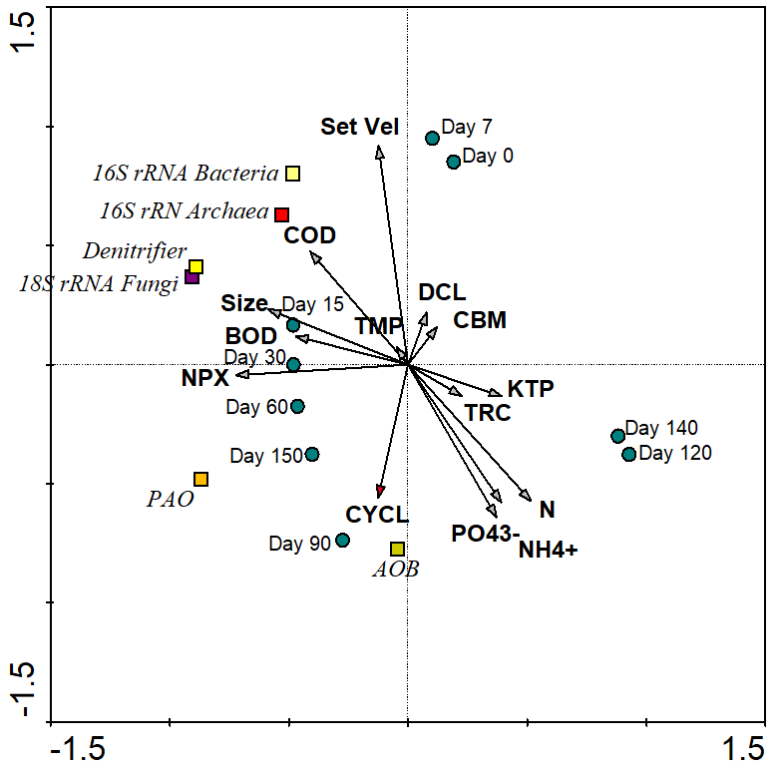

Figure 7. Linkage of the number of gene copies with physico-chemical performance and drug degradation using multivariate redundancy analysis.

The RDA demonstrated that TRC and KTP were negatively correlated with the abundances of *Bacteria*, *Archaea*, *Fungi* and, more specifically, with denitrifier, while phosphate accumulating bacteria and ammonia oxidating bacteria had not correlation. The *amoA* gene demonstrated a strong positive correlation with high CCLP removal, which

was only detected during the first 15 days. Interestingly, TMP showed no correlation with the number of 16S rRNA genes of *Bacteria*, although it is an antimicrobial compound with bactericidal action.

Antibiotics could change the microbial community structure, functioning or composition, and bactericides could promote the disappearance of some phylotypes and alter the bacterial community (Danner et al., 2019). In another study, phosphate removal from raw WW was not linked to the abundance of PAO, possibly because of the inhibition of their metabolism (Correa-Galeote et al., 2021). In contrast, the results highlighted the positive correlation of ammonia oxidation with *amoA* genes. Higher COD and BOD removal rates were strongly and positively correlated with the abundances of *Bacteria*, *Archaea*, *Fungi* and, more specifically, denitrifiers. As denitrifying microorganisms usually use the organic matter as a carbon source, this can be explained by the ubiquitous heterotrophic denitrifying activity (M. Hurtado-Martinez et al., 2021).

The RDA performed for archaeal, bacterial and fungal populations (Figures S8A, S8B, S8C, respectively) related to the physico-chemical parameters demonstrated that all studied communities changed progressively over time, despite the toxicity and oscillations of the raw water; interestingly, the community was stable from days 90 to 150. The communities showed a positive intercorrelation on days 90, 120, 140 and 150, highlighting the robustness of the granular biofilm regardless of the influent composition.

In the RDA performed for archaeal dynamics, two compounds (TRC, KTP) were positively correlated with the steady-state period, whereas four (TMP, DCL, CBM, NAP) were positively correlated with the first month of operation. In this way, the dominant OTUs from the inoculum showed a reduced evenness, with a massive proliferation of OTUs 01 and 03, both belonging to *Bathyarchaeota*. Despite the overgrowth of OTU 01 and OTU 03 during the first month of operation, only OTU 01 was correlated with TMP and DCL removal. On the other hand, the high abundance achieved by OTU 02 from day 30 could be related to the resistance to PhACs, with a strong and positive correlation with KTP and TRC as well as with nitrogen and phosphate removal. The OTU 02, taxonomically affiliated to *Methanocaldococcus* is known by its implication in the nitrogen biogeochemical cycle. Recently, this genus has attracted increased interest because of its role in diazotrophic metabolism and its implications for the origin of nitrogenase (Payne et al., 2023; Vo et al., 2023).

The huge abundance of dominant bacterial OTUs prevents a clear recognition of the role of each of them in the performance of the reactor. Overall, 69 OTUs were correlated with a good performance in terms of TMP, NPX, CYCL, TRC, KTP, BOD, NH_4^+, PO_4^{3-} and N removal, of which only 20 OTUs were correlated with the steady-state on days 90, 120, 140 and 150; these 20 OTUs were exclusively linked with N, KTP, TRC, NH_4^+, $PO_4^{3-}$and BOD removal. The granular properties were negatively intercorrelated, indicating that larger granules settle more slowly. It has therefore been assumed that a better settling velocity is related to the compactness and density of the granules (Hamza et al., 2022).

The RDA of the fungal community corroborates the ability of fungal phylotypes to growth on biofilm, and results demonstrate how granules act as an ecological niche with a large fungal diversity. This implies that fungi play an essential role in the structural core and granulation processes of this biotechnological approach (Munoz-Palazon et al., 2020a; 2020b; Han et al., 2022). In this study, 23 OTUs were positively correlated with a high drug removal efficiency throughout the experimental period.

4. CONCLUSIONS

To date, no study has demonstrated the ability of granular aerobic systems to reduce PhACs concentrations in real HWW. This study demonstrates the robustness and stability of granular biomass for the treatment of real hospital WW, regardless of the characteristics of the influent. BOD_5 and nitrogen removal were high (100 % and 70 % - 90 %, respectively), whereas phosphate removal reached 50 % of removal. The results highlight the ability of the system to degrade TMP, CMP, CLP and TRC at high influent loads, whereas release peaks were suspected for NSAIDs. The PhACs modified the bacterial and fungal populations, increasing diversity and evenness due to competition between them. The dynamics of the archaeal population showed a progressive adaptation phase. The absolute abundance of genes was not significantly affected, except for PAO and fungal organisms. Despite the larger granule size and the decrease in settling ability over time, the stability of the granular structure was not compromised. The aerobic granular sludge technology is a feasible technology for treating hospital WW in order to reduce the contaminant load prior to disposal to the urban WW.

Credit authorship contribution statement

Lizandra Perez-Bou: Data curation, Investigation, Writing – review & editing. Aurora Rosa-Masegosa: Conceptualization, Data curation, Formal analysis, Resources,

Supervision, Writing – original draft. Ramiro Vilchez-Vargas: Formal analysis, Funding acquisition, Investigation, Software, Writing – review & editing. Alexander Link: Funding acquisition, Resources, Writing – review & editing. Alejandro Gonzalez-Martinez: Supervision, Writing – review & editing. Jesus Gonzalez-Lopez: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing. Barbara Munoz-Palazon: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Supervision, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Data availability Data will be made available on request.

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SUPPLEMENTARY MATERIAL

Treatment of hospital wastewater using aerobic granular sludge technology: removal performance and microbial dynamics

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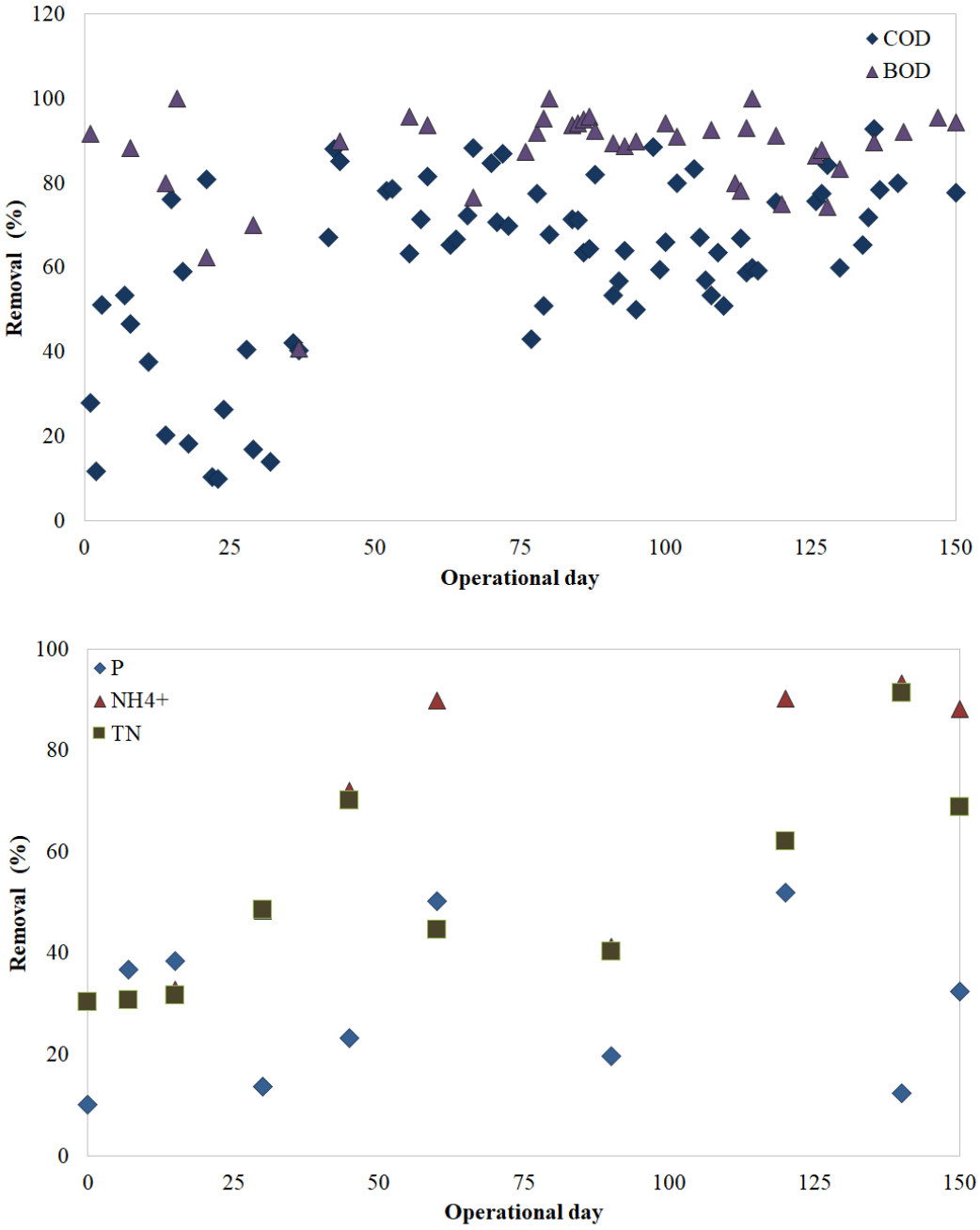

Figure S1. Removal efficiency ratio of COD (%), BOD_5(%) and nutrients removal (%).

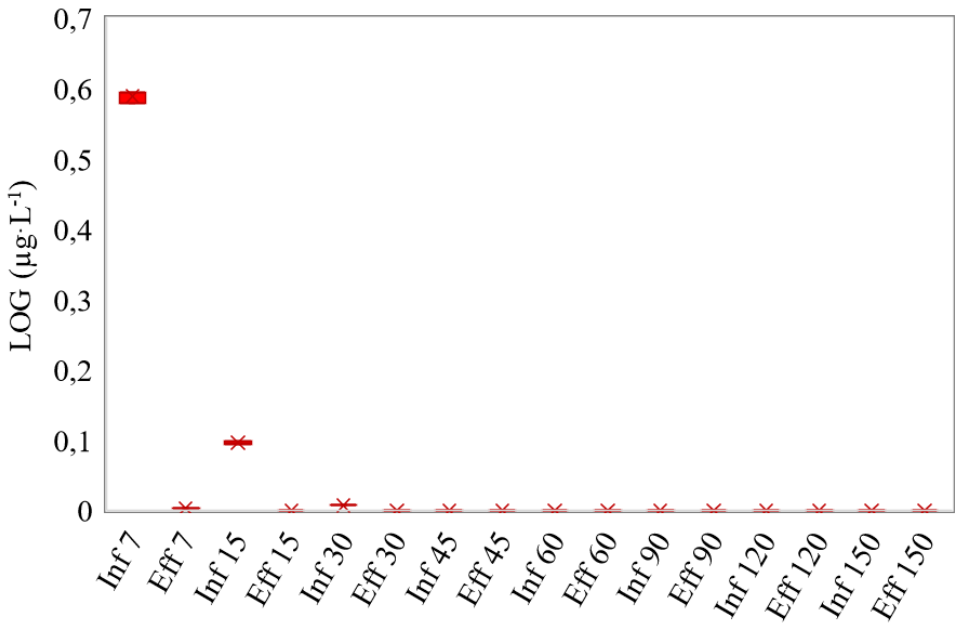

Figure S2. Cyclophosphamide analysis measured by UHPLC detected only in three influent samples at day 7, day 15, day 30.

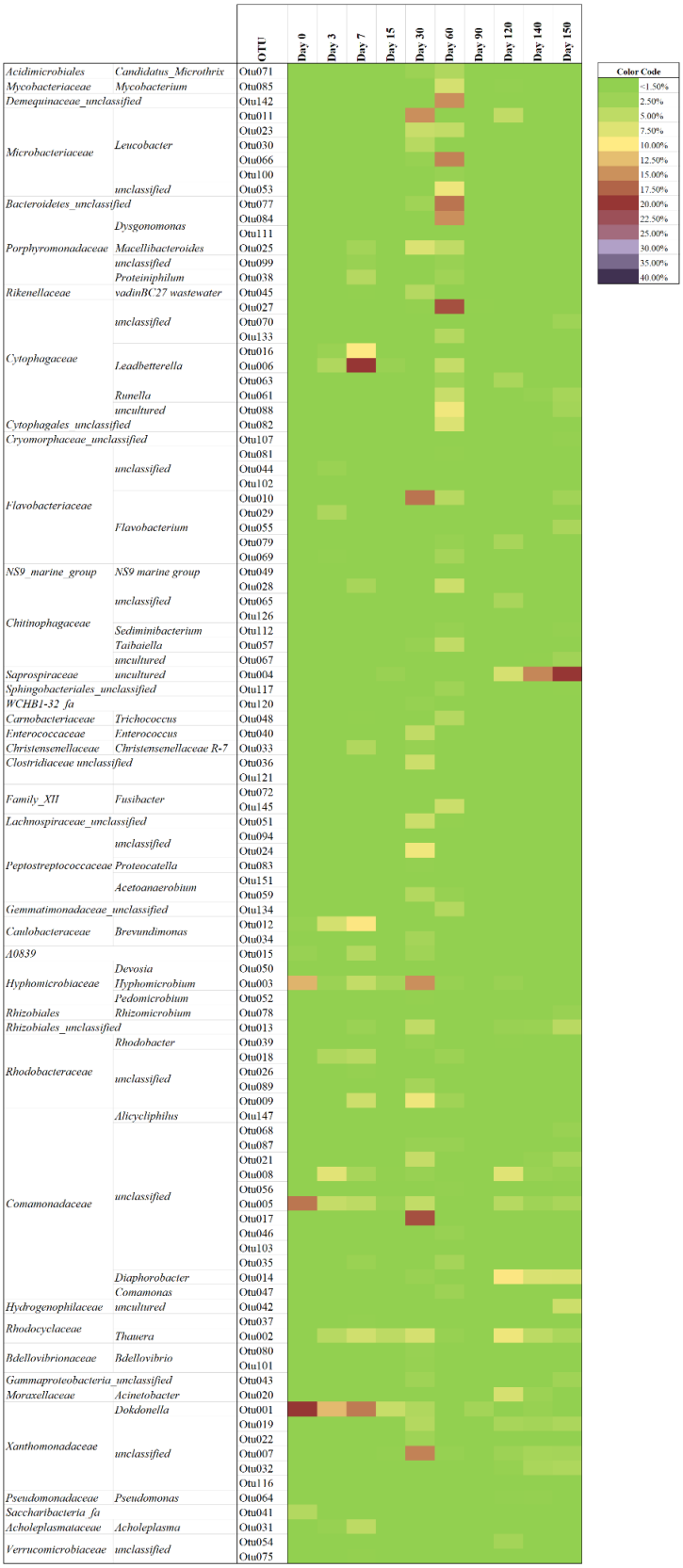

Figure S3. Heat of map of bacterial dynamics expressed as a taxonomic operational unit (OTU) with phylogenetic similarity of 97% with more than 1.50% of relative abundance.

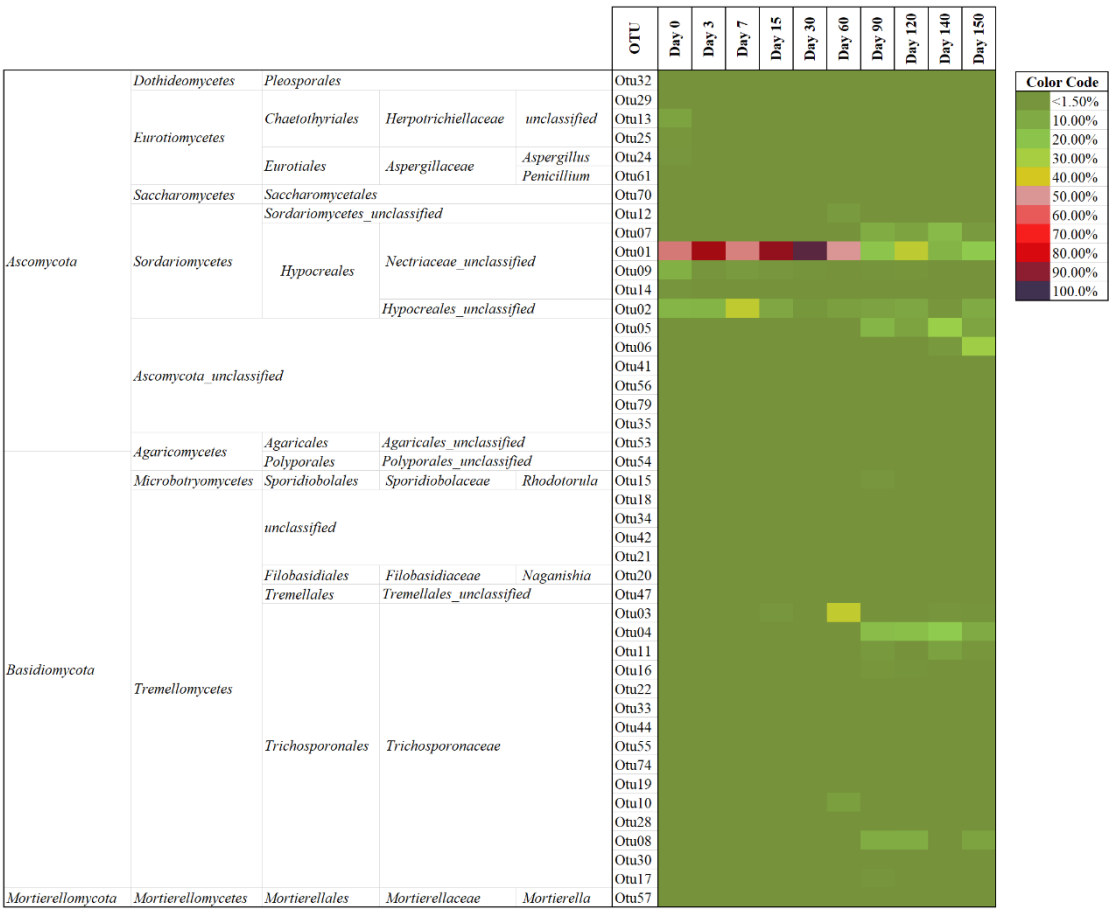

Figure S4. The heat of map of fungal dynamics expressed as a taxonomic operational unit (OTU) with phylogenetic similarity of 95% with more than 1.50% of relative abundance.

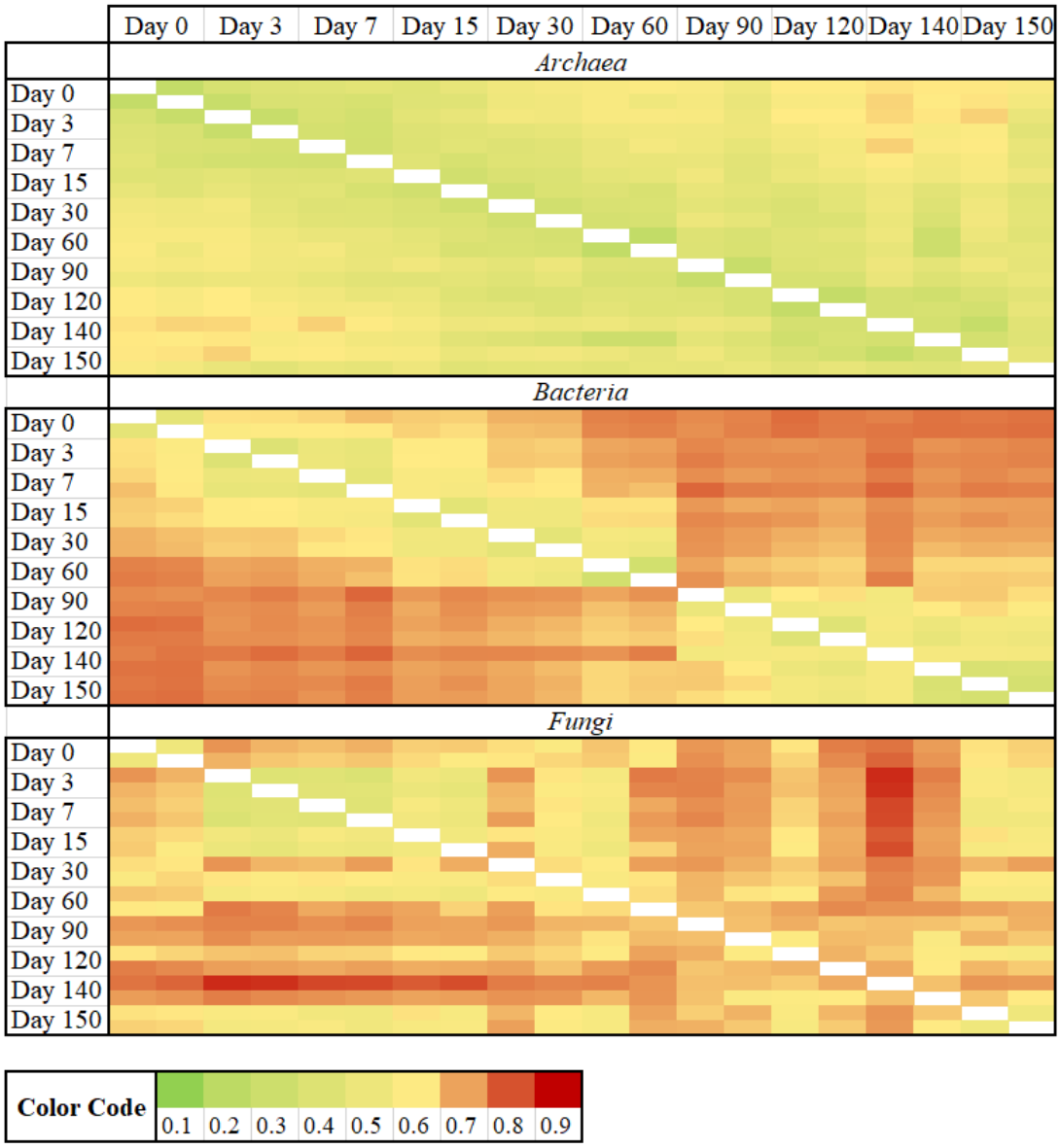

Figure S5.β-diversity measured by Whittaker index between pairs of samples for *Archaea*, *Bacteria* and *Fungi* communities.

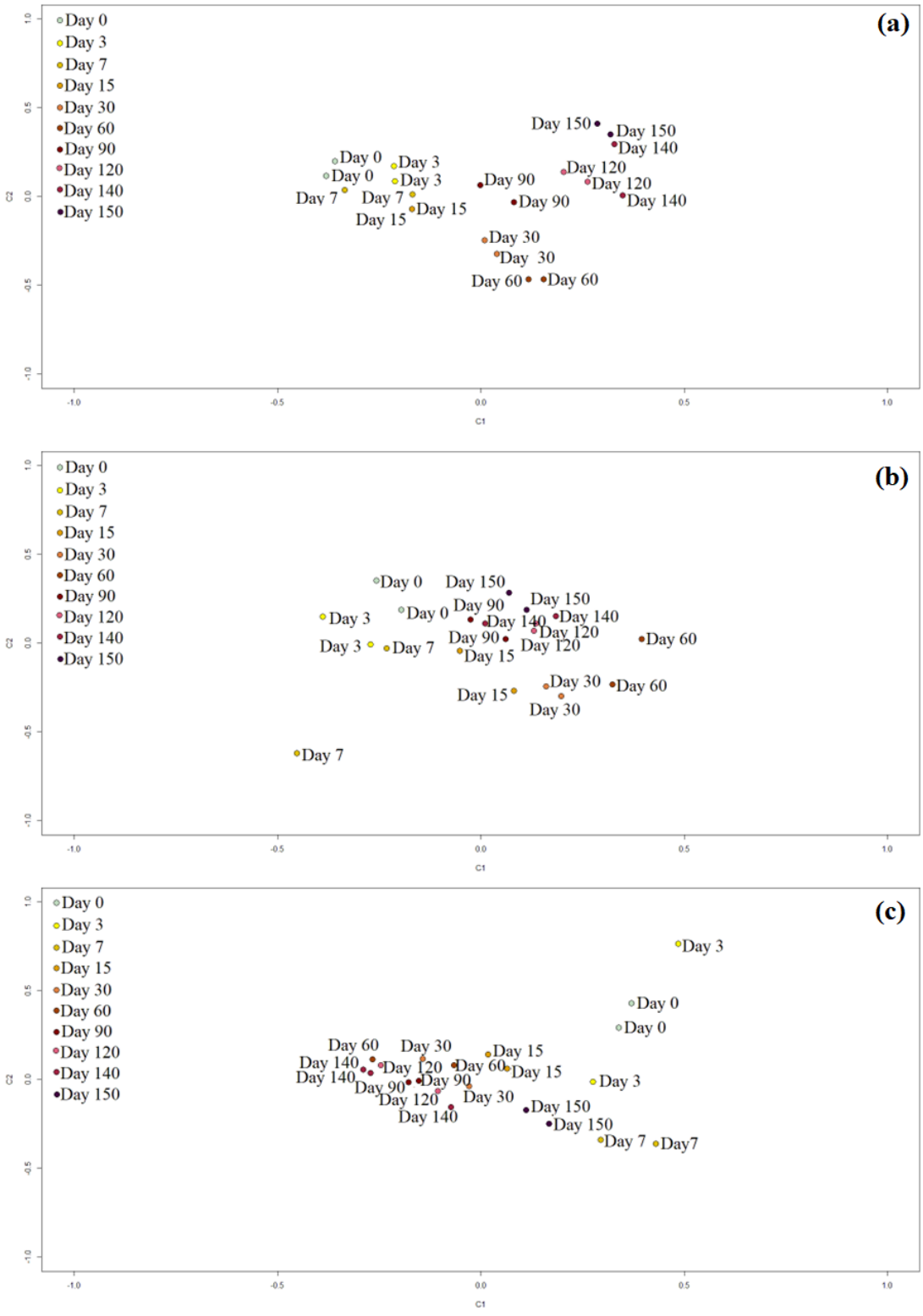

Figure S6. Principal Component Analysis (PCA) calculated with compositional statistical of duplicate samples from next-generation sequencing for *Archaea* (a), *Bacteria* (b) and *Fungi* (c).

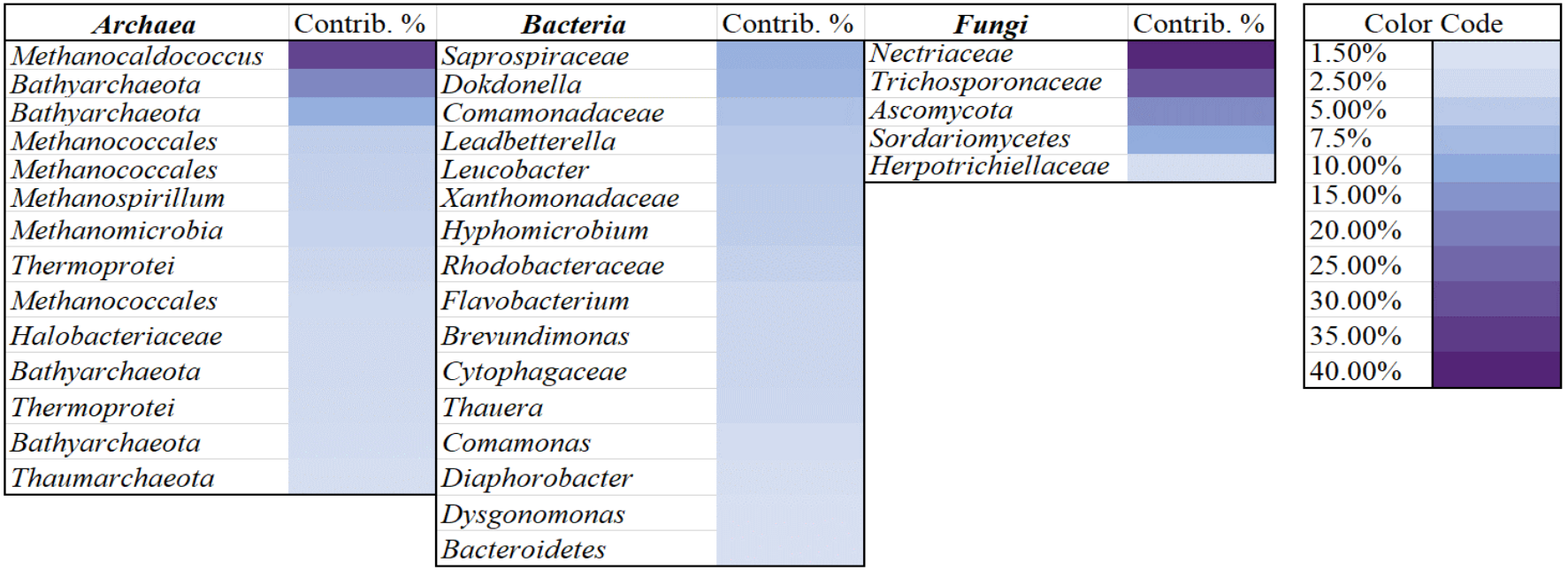

Figure S7. Similarity percent analysis (SIMPER) calculated to elucidate the contribution of genera (<1.5%) to dissimilarities between start-up and steady-state phases for *Archaea*, *Bacteria* and *Fungi.*

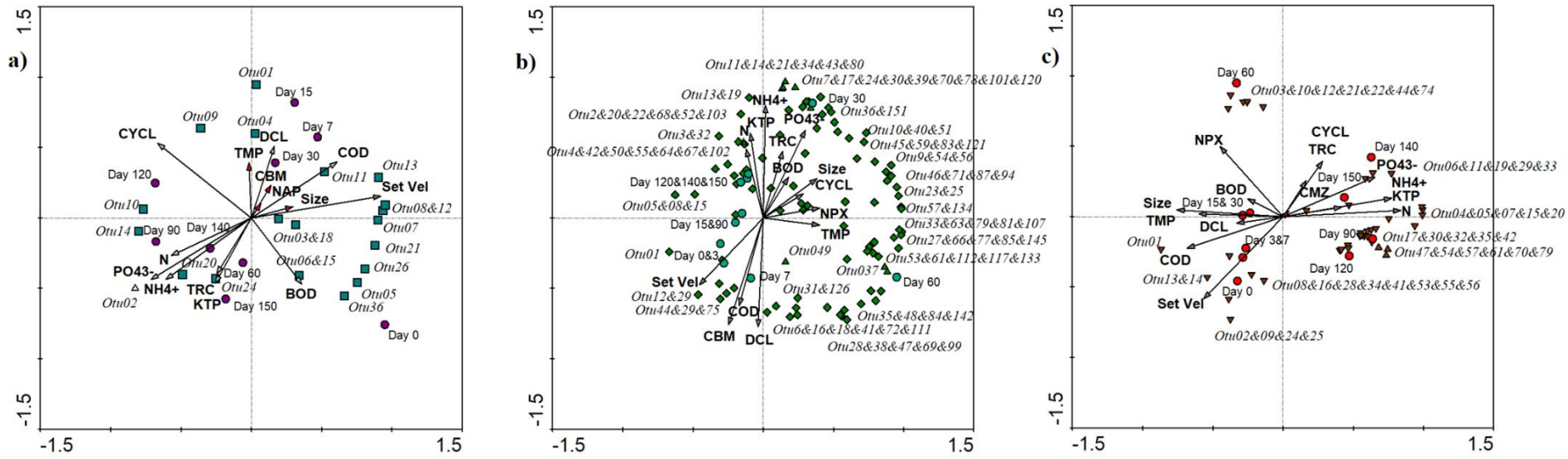

Figure S8. RDA analysis of archaeal (a), bacterial (b), and fungal (c) dominant OTUs with the performance of the reactor (PhACs, COD, BOD_5, Nitrogen, ammonium, and phosphate removal), and granular properties.

Table S1. PERMANOVA analysis for the evaluation of startup (day 0 to day 38) and steady-stable period (day 39 to day 150).

PERMANOVA	Nitrogen	Phosphate	BOD rem	COD rem	Settle	Size
Permutation N:	9999	9999	9999	9999	9999	9999
Total sum of squares:	0,2988	1,837	0,2542	3,15E+04	0,3686	0,8804
Within-group sum of squares:	0,1446	0,9366	0,1982	2,16E+04	0,1417	0,8107
F:	8,53	7,69	9,614	32,92	40,04	2,149
p (same):	0,0226	0,0063	0,0051	0,0001	0,0001	0,1488

Table S2. Alpha diversity of archaeal, bacterial and fungal communities over operational days.

		Day 0	D.S	Day 3	D.S	Day 7	D.S	Day 15	D.S	Day 30	D.S	Day 60	D.S	Day 90	D.S	Day 120	D.S	Day 140	D.S	Day 150	D.S
Archaea	Simpson	0,9	0,0	0,8	0,0	0,9	0,0	0,5	0,0	0,9	0,0	0,8	0,0	0,8	0,0	0,8	0,0	0,8	0,0	0,7	0,2
	Shannon	3,0	0,0	2,4	0,1	2,8	0,1	1,5	0,1	2,8	0,0	2,6	0,0	2,2	0,0	2,3	0,0	2,6	0,1	2,4	0,8
	Evenness	0,4	0,1	0,2	0,0	0,3	0,0	0,1	0,0	0,2	0,0	0,1	0,0	0,1	0,0	0,1	0,0	0,1	0,0	0,1	0,1
	B-Parker	0,1	0,0	0,4	0,0	0,2	0,0	0,7	0,0	0,3	0,0	0,5	0,0	0,5	0,0	0,3	0,0	0,4	0,0	0,5	0,3
	Chao	60,6	14,1	116,5	34,7	114,8	26,5	161,6	21,2	167,6	12,6	157,5	6,9	92,0	4,2	151,9	9,1	190,5	10,8	173,7	3,2
Bacteria	Simpson	0,9	0,0	1,0	0,0	1,0	0,0	0,9	0,0	1,0	0,0	1,0	0,0	1,0	0,0	1,0	0,0	1,0	0,0	0,9	0,0
	Shannon	3,6	0,1	4,4	0,0	4,5	0,2	3,4	0,0	4,5	0,1	5,0	0,0	5,0	0,2	5,0	0,1	4,8	0,1	4,4	0,0
	Evenness	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,1	0,0	0,3	0,1	0,1	0,0	0,2	0,1	0,1	0,0
	B-Parker	0,2	0,0	0,1	0,0	0,2	0,0	0,2	0,0	0,1	0,0	0,1	0,0	0,0	0,0	0,1	0,0	0,1	0,0	0,2	0,0
	Chao	2014,0	107,5	3643,0	135,8	4195,5	734,7	3526,5	265,2	4163,5	71,4	4036,5	416,5	1010,8	346,8	2208,5	135,1	1713,2	1087,3	2258,5	75,7
Fungi	Simpson	0,6	0,1	0,3	0,0	0,6	0,0	0,3	0,0	0,1	0,0	0,7	0,0	0,9	0,0	0,8	0,0	0,8	0,0	0,8	0,0
	Shannon	1,6	0,3	0,8	0,0	1,4	0,0	0,8	0,0	0,3	0,0	1,4	0,0	2,5	0,2	2,2	0,1	1,9	0,2	2,2	0,2
	Evenness	0,1	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,0	0,2	0,0	0,1	0,0	0,1	0,0	0,1	0,0
	B-Parker	0,5	0,1	0,8	0,0	0,5	0,0	0,8	0,0	1,0	0,0	0,5	0,0	0,2	0,0	0,3	0,0	0,3	0,0	0,3	0,0
	Chao	291,8	38,5	666,5	67,9	625,1	78,1	526,2	85,5	476,8	147,9	278,9	159,5	153,4	10,4	228,8	18,0	137,7	57,1	367,4	19,6

*D.S: Deviation standard; B-Parker: Berger-Parker; Evenness : Pielou's evenness.

CHAPTER-II

Abstract

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Perez-Bou L, Gonzalez-Martinez A, Cabrera JJ, Juarez-Jimenez B, Rodelas B, Gonzalez-Lopez J, Correa-Galeote D. Design and Validation of Primer Sets for the Detection and Quantification of Antibiotic Resistance Genes in Environmental Samples by Quantitative PCR. Microbial Ecology. 2024. 87(1):71.

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Scientific quality index:

Cite score: 6.9

Impact factor: 3.3

The high prevalence of antibiotic resistant bacteria (ARB) in several environments is a great concern threatening human health. Particularly, wastewater treatment plants (WWTPs) become important contributors to the dissemination of ARB to receiving water bodies, due to the inefficient management or treatment of highly antibiotic-concentrated wastewaters. Hence, it is vital to develop molecular tools that allow proper monitoring of the genes encoding resistances to these important therapeutic compounds (antibiotic resistant genes, ARGs). For an accurate quantification of ARGs, there is a need for sensitive and robust qPCR assays supported by a good design of primers and validated protocols. In this study, eleven relevant ARGs were selected as targets, including *aadA* and *aadB* (conferring resistance to aminoglycosides); *ampC*, bla_{TEM}, bla_{SHV}, and *mecA* (resistance to beta-lactams); *dfrA*1 (resistance to trimethoprim); ermB (resistance to macrolides); *fosA* (resistance to fosfomycin); *qnrS* (resistance to quinolones); and *tetA*(A) (resistance to tetracyclines). The *in silico* design of the new primer sets was performed based on the alignment of all the sequences of the target ARGs (orthology grade>70%) deposited in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, allowing higher coverages of the ARGs' biodiversity than those of several primers described to date. The adequate design and performance of the new molecular tools were validated in six samples, retrieved from both natural and engineered environments related to wastewater treatment. The hallmarks of the optimized qPCR assays were high amplification efficiency (>90%), good linearity of the standard curve (R^2>0.980), repeatability and reproducibility across experiments, and a wide linear dynamic range. The new primer sets and methodology described here are valuable tools to upgrade the monitorization of the abundance and emergence of the targeted ARGs by qPCR in WWTPs and related environments.

Key words: antibiotic resistance genes, primer design, assay optimization, qPCR, antibiotics, WWTPs

CHAPTER II: Design and validation of primer sets for the detection and quantification of antibiotic resistance genes in environmental samples by quantitative PCR

1. INTRODUCTION

The antibiotic resistance phenomenon is one of the biggest issues of concern for human health in the twenty-first century. Controlling the dissemination of antibiotic resistance genes (ARGs) in the environment is a key challenge to guarantee the longevity of the therapeutic capacity of these important pharmaceuticals (Omuferen et al., 2022). Once released into the environment, antibiotic compounds cause the enrichment of antibiotic resistant bacteria (ARB), even in sub-inhibitory concentrations (Slipko et al., 2021). Therefore, the widespread dissemination of ARB and the proliferation of ARGs and their potential mobilization through horizontal gene transfer (HGT) requires a reliable determination of their occurrence and abundance in order to improve the understanding of their dynamics in environmental hotspots. Wastewater treatment plants (WWTPs) are important reservoirs of ARGs via inefficient management or treatment of highly antibiotic-concentrated wastewaters, contributing to the dissemination of both ARGs and ARB to receiving water bodies (Keenum et al., 2022). A lack of clear guidelines for acceptable levels of antibiotic, ARB, and ARG pollution warrants the design of assays for their quantification in environmental samples to prevent the spread of ARB. In this regard, the necessity to monitor the clinically and anthropogenically relevant ARGs has been highlighted by several governmental entities, i.e., the U.S. Centers for Disease Control and Prevention (https://www.cdc.gov/drugresistance/index.html), the National Aquatic Resource Survey of the U.S. Environmental Protection Agency (Keely et al., 2022) and the Antimicrobial Resistance Surveillance System of the European Centre for Disease Prevention and Control (https://www.ecdc.europa.eu/en/antimicrobial-resistance).

Currently, ARGs' monitorization relies on the use of highly accurate molecular-based methods, displacing progressively the time- and money-consuming culture-based approaches (Keenum et al., 2022). In this respect, quantitative polymerase chain reaction (qPCR) has become the gold standard method for the detection and quantification of

ARGs in environmental samples, due to technical advantages such as faster results, more specific detection, and the user-friendly methodology (Keenum et al., 2022). A plethora of research addressed the abundance of the different ARGs in environmental samples by qPCR, including wastewater (WW) (Slipko et al., 2021), activated sludge (Wei et al., 2018), and WW impacted surface water (Eramo et al., 2019), soil samples and manure (Xu et al., 2019). In general, the success of a qPCR assay requires appropriately validated primers and correct standards, together with the optimization of the assay performance according to the minimum information for publication of quantitative real-time PCR experiments (MIQE guidelines) (Bustin et al., 2009). Frequently, these parameters are not optimized enough, which could result in non-specific amplification or under quantification of the targets leading to inaccurate and imprecise results (Bustin and Huggett, 2017). In this regard, to obtain the highest accuracy of the abundance measures of a specific target gene during qPCR assays, the proper design of the corresponding primers stands out as the most important factor (Bustin and Nolan, 2017). Literature regarding quality primer pair design describes several significant properties to consider, mainly, the primer size, the percentage of guanine and cytosine, the lack of formation of secondary structures, and an adequate range of annealing temperatures (Bustin, 2009; Dreier et al., 2020). However, some developed qPCR assays are inadequately designed and do not meet these key quality criteria (Bustin and Nolan, 2017).

On the other hand, most previous research has been focused on the characterization of individual pathogens or specific groups within a taxon. This approach restricts the design of universal primers enabling the detection and quantification of the broad genetic divergences for the corresponding antibiotic resistance mechanism (Tremblay et al., 2021). In addition, it is necessary to continuously update the available molecular tools to avoid the miscalculation of ARGs' abundance in the environment, which could lead to the underestimation of the extent of their dissemination and the potential for their acquisition by previously sensitive bacteria. Environmental monitoring of ARGs using qPCR requires primer sets useful for the analysis of a wide range of target bacteria in different types of environmental samples, and the main parameters that need to be addressed are the assay's analytical sensitivity and specificity (Bustin et al., 2009). Therefore, this study describes the development of new primer sets aimed at amplifying a broader diversity of the ARGs *aadA*, *aadB*, *ampC*, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM}}$, *dfrA*1, *ermB*, *fosA*, *mecA*, *qnrS*, and *tetA*(A). The selection of these ARGs was based on the reported clinical

importance and incremented resistance to the corresponding antibiotics in WWTPs (Wang et al., 2020; Nguyen et al., 2021). The enzymes encoded by the *aadA* and *aadB* genes confer resistance to aminoglycosides (gentamicin, hygromycin B, kanamycin, neomycin, spectinomycin, and tobramycin), the sixth most commonly used antimicrobial class in veterinary medicine in Europe (van Duijkeren et al., 2019). These genes are frequent pathogens with extensive resistance to antibiotics, many of which are detected in municipal WW (Selvaraj et al., 2022). The *ampC* gene and the bla_{SHV} and bla_{TEM} genes encode AmpC beta-lactamases and extended-spectrum beta-lactamases (ESBL), respectively, which can inactivate most broad-spectrum beta-lactam antimicrobials (third-generation cephalosporins, penicillins, and aztreonam), with the exception of cefepime and carbapenems (Mohamed et al., 2020). Similarly, the *mecA* gene encodes for a penicillin-binding protein (PBP2a), which confers resistance to all beta-lactam compounds (Liao et al., 2018). The expression of the *dfrA*1 gene inhibits the therapeutic effect of the combination of trimethoprim/sulfamethoxazole. These antibiotics are poorly removed during WW treatment (Sánchez-Osuna et al., 2020). The presence of *ermB* confers resistance to macrolides and it is also frequently associated with resistance to lincosamide and type B streptogramin, resulting in treatment failure to these three antibiotics groups, which inhibit bacterial protein synthesis (Timsina et al., 2020). The enzyme encoded by the *fosA* gene confers resistance to fosfomycin, an antibiotic amply released into the environment via WW, as it is routinely used for the treatment of urinary infections caused by extensively drug-resistant (XDR) Gram-negative bacteria (Zurfluh et al., 2020). The *qnrS* gene mediates the resistance to quinolones, an antimicrobial resistance of the highest priority due to its significance in human medicine, particularly in developing countries (Castrignanò et al., 2020). This gene is frequently detected in natural environments since most WWTPs only remove a small amount of this antibiotics (Dai et al., 2022). Finally, the *tetA* gene class A (*tetA*(A)) is the molecular marker of the resistance of tetracyclines according to its abundance and relationship with anthropogenic inputs (Nnadozie et al., 2019), whose broad presence in the environment is related to the nearly universal use of tetracyclines in livestock production (Møller et al., 2016).

The molecular tools and the new qPCR protocols here developed were validated using DNA extracted from different environmental samples, including activated sludge, river sediment, and agricultural soils, ecosystems previously described as hotspots of the

dissemination of the targeted ARGs and as environmental reservoirs of ARB (Marti et al., 2013 a,b; Zhang et al., 2020a; Schages et al., 2021; Omuferen et al., 2022).

2. MATERIALS AND METHODS

2.1 *In silico* design and validation of primer sets

The specific DNA sequences of the target genes *aadA*, *aadB*, *ampC*, *bla*$_{TEM}$, *bla*$_{SHV}$, *mecA*, *dfrA*1, *ermB*, *fosA*, *qnrS*, and *tetA*(A) were retrieved from the Kyoto Encyclopedia of Genes and Genomes (KEGG, https://www. genome.jp/kegg/), including all sequences with an orthology grade > 70% for a given KEGG orthology number (Table 1SI). The KEGG database entries employed for the present study are listed in Table 1. The sequences were aligned using the MAFFT algorithm, and the *in silico* design of the primers was performed using the Geneious 2021.1.1 software (Biomatters, Auckland, New Zealand). The specificity of the candidate primers over the target regions was assessed by querying the full genome (chromosomes and plasmids) of each of the strains included in this study (listed in Table 2SI), to ensure the absence of non-specific annealing outside of the target DNA fragment. The complete genomes were retrieved from the GenBank DNA database (https://www.ncbi.nlm.nih.gov/genbank/). Finally, the *in silico* validated oligonucleotides were synthesized by the company Sigma-Aldrich (Merck, Germany).

Table 1. KEGG database entries of sequences for each ARGs selected.

Gene Target	KEGG database entries
aadA	K00984: streptomycin 3”-adenylyltransferase
aadB	K17881: aminoglycoside 2"-adenylyltransferase
ampC	K01467: beta-lactamase class C
bla$_{TEM}$	K18698: beta-lactamase class A TEM
bla$_{SHV}$	K18699: beta-lactamase class A SHV
*dfrA*1	K18589: dihydrofolate reductase
ermB	K00561: 23S rRNA (adenine-N6)-dimethyltransferase
fosA	K21253: glutathione S-transferase fosA
mecA	K02545: penicillin-binding protein 2 prime
qnrS	K18555: fluoroquinolone resistance protein
tetA(A)	K08151: DHA1 family, tetracycline resistance protein

2.2 Optimization of PCR thermocycling profiles and construction of plasmids standards for ARGs quantification by qPCR

To optimize the conditions from PCR amplification of the target ARGs, both genomic DNA from selected culture strains carrying the genes and a pool of DNA previously isolated from activated sludge samples were used as templates. The reference bacterial strains used in this study were purchased from the Spanish Type Culture Collection and cultivated in the corresponding media listed in Supplementary Table 1 SI. The total DNAs of the five reference bacterial strains were isolated using a FastDNA kit (QBio/MP Biomedicals, LLC, France) according to the manufacturer instructions. Amplification reactions were implemented in a final volume of 25 µL, comprising 2.5 µL of 10×DreamTaq buffer (Thermo Scientific, USA), 0.5 µL of True Pure dNTPs (8 mM) (Canvax, Spain), 0.15 µL of each primer (10 µM), 0.125 µL of 5 U/µL DreamTaq Hot Start DNA Polymerase (Thermo Scientific, USA), 0.125 µL of dimethyl sulfoxide (Sigma-Aldrich, USA), 0.0625 µL of 20 mg/mL of bovine serum albumin (New England Biolabs, USA), 19.3875 µL of ultrapure water, and 2 µL of template DNA. The thermocycling profiles of the reactions were optimized for linearity, sensitivity, specificity, repeatability, and reproducibility. The size of the PCR amplification products was checked by electrophoresis in 2% agarose gels.

For the construction of plasmids standards for ARG quantification, the amplicons were purified using the QIAquick PCR purification kit (QIAGEN, Germany). The purified PCR products were ligated into the PCR 4-TOPO vector (Invitrogen, USA) and then used to transform *Escherichia coli* DH5α competent cells according to the specifications of the TOPO TA cloning system (Invitrogen, USA). Subsequently, eight transformant colonies obtained in each reaction were randomly selected to verify the presence of an insert of the right size by PCR, using agarose gel electrophoresis. Finally, four positive plasmids were selected for each ARG to confirm the correct identity of the inserts by means of Sanger sequencing in the facilities of the Genetic Information Unit of the Scientific Instrumentation Center (University of Granada, Spain). Sequences sharing>98% identity with the corresponding control sequences were subsequently used as standards.

2.3 Environmental samples and DNA extraction

This study included the following types of environmental samples: activated sludge, composting sludge, anaerobic digestion sludge, agricultural soil, and river sediment. Two

different activated sludge samples were collected from the aeration tank in the secondary treatment step of the biofactories "Churriana de la Vega-Sur" (AS-CHU, UTM coordinates 30N 44509, 13,343) and "Los Vados" (AS-VA, UTM coordinates 30N 39964, 16,334), Granada, Spain. Composting sludge (COM) was collected from sewage sludge composting performed in the set of the environmental complexes Ecoindustria del Reciclado (EIDER) (Guadix, Granada, Spain, UTM coordinates 30N 92646, 30,931). The biomass of anaerobic digestion sludge (AD) employed in this study was collected from an operational bioreactor producing volatile acids from the olive residue (alperujo) from Instituto de la Grasa (Seville, Spain, UTM coordinates 30N 39770, 38,833). The river sediment (SED) was obtained from the Genil River in Granada City, Spain (UTM coordinates 30 N 43940, 13,314), in which the effluents generated in the AS-CHU are discharged. The agricultural soil (SOIL) was retrieved from a farming field irrigated by the Genil River (UTM coordinates 30N 444331, 4,114,407). All samples were immediately frozen after collection and stored at -20 °C until DNA extraction.

Total DNA from the environmental samples (500 mg) was extracted using the FastDNA-2mL SPIN Kit for Soil and the FastPrep24 apparatus (MP-BIO, USA), according to the manufacturer instructions. Three independent biological replicates were used from each sample. The quality and concentration of the extracted DNA were measured by spectrophotometry using NanoDrop 2000 (Thermo Scientifc, USA) and verified by electrophoresis on 1% agarose. DNA samples were stored at -20 °C for further use

2.4 Quantification assays

Quantification of the target ARGs in environmental samples was performed by qPCR on a QuantStudio-3 Real-Time -PCR system (Applied Biosystems, USA), using the same reaction conditions as those described in the "Optimization of PCR Thermocycling Profiles and Construction of Plasmids Standards for ARGs Quantifcation by qPCR" section for conventional PCR, employing SYBR-Green I (0.125 µL, 20×SYBR Green I (Thermo Scientific, USA)) as dye method for real-time fluorescence monitoring. Serial dilutions of linearized plasmids containing the target genes, ranging from 10^8 to 10 copies/µL, were used to construct the standard curves for the absolute quantification. The general workflow employed in this study is summarized in Figure. 1.

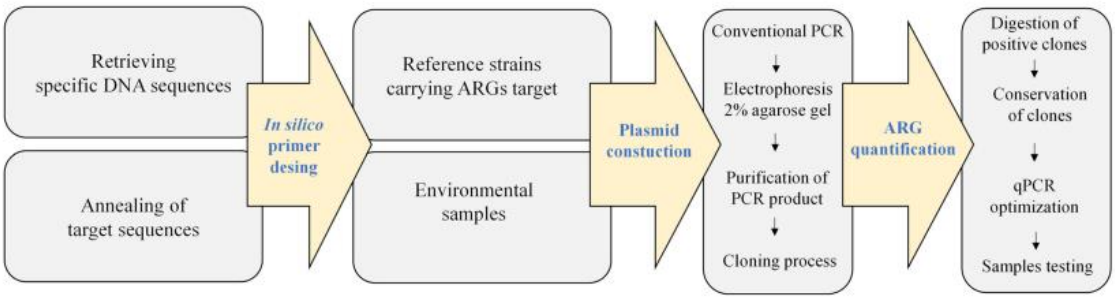

Figure. 1 Workflow diagram used in this study for the design and validation of new pairs of primers for ARGs quantification by qPCR.

Samples were run in three independent biological and technical replicates along with non-template controls in two different assays (n=18), to evaluate the repeatability and reproducibility of the qPCR protocols. The number of total copies of the ARGs was expressed per gram of environmental sample, according to the predicted concentration of the corresponding standard amplification plots (Correa-Galeote et al., 2013). In addition, the relative abundance of ARGs was calculated as the normalized ratio of the number of ARGs copies to the corresponding number of genes encoding 16S rRNA copies, which were measured in all samples following a previously described protocol (Correa-Galeote et al., 2021).

2.5 Statistical analysis

The statistical differences among data sets from different samples were analyzed using the non-parametric Kruskal-Wallis and Conover Iman tests ($p < 0.05$ significance level) in XLSTAT v2020 (Addinsoft, USA).

3. RESULTS AND DISCUSSION

3.1 *In Silico* primer design and ARG diversity

Sequences of the target ARGs with orthology degrees higher than 70% were retrieved from the corresponding KEGG entries (*aadA*: 96 sequences, *aadB*: 21, *ampC*: 81, *bla*$_{SHV}$: 89, *bla*$_{TEM}$: 81, *dfrA*1: 42, *ermB*: 58, *fosA*: 20, *mecA*: 37, *qnrS*: 15, *tetA*(A): 49) (Table 1SI). Subsequently, alignments were conducted using the MAFFT algorithm implemented in the Geneious Prime software to perform the *in silico* primer design. This process was made following the recommendations based on coverage and specificity criteria of Dreier et al. (2020), the identification of a specific target nucleotide sequence, and the later design of primers that bind exclusively to the target sequences.

According to the parameters suggested by Bustin and Huggett (2017), the adequate annealing temperatures (50-65 °C), base composition (GC content ranging of 50-60%), length (between 18 and 25 pb), absence of secondary structure, lack of potential hairpin formation, and lack of self-annealing were accomplished. To guarantee the total *in silico* coverage of the newly designed primers on the template sequences, degenerated bases were introduced into the primers to match all the sequences (maximum 2 degenerations per primer) wherever necessary. The new primer sets guaranteed the total coverage of the template sequences (allowing two ambiguities per primer), generating amplicon sizes between 215 and 494 bp. In addition, the specificity of amplification of the new primers was tested in the 557 chromosomal and plasmid genomes used in this study, without obtaining *in silico* annealings out of the genetic zones of the genomes previously described as antibiotic resistance coding sequences.

The candidate primers that matched over every targeted sequence and only exclusively over the target regions were considered for further research. The sequences of the new primers designed here and their main features are presented in Table 2. Higher coverages of the newly developed primers for the quantification of the selected sequences of *aadA*, *ampC*, *bla*$_{SHV}$, *dfrA*1, *mecA*, *qnrS*, and *tetA*(A) genes were found, compared to those of all the previously available primers tested in this study (Table 3SI). Additionally, most primers formerly described for the *fosA* gene also presented very low coverages, except those developed recently by Abbott et al. (2020). However, the presence of a triplet at the beginning of the forward primer and a higher level of homodimers in the reverse primer is detrimental compared to the new primer set (fosA-7F/fosA-306R) designed in this study. On the other hand, several of the available primers for *aadB*, *bla*$_{TEM}$, and *ermB* presented coverages of 100% over the selected sequences; however, a large number of non-compliances with the recommendations of the MIQE guidelines were found for these primers, i.e., production of secondary structures of the amplicon sequence, high potential to form primer dimers, and triple repeats of the same nucleotide. Therefore, this underlines the necessity to develop new ones that solve those issues, facts that were minimized in the new primers designed. In this sense, Keenum et al. (2022) recently reported poor coverages of some of the most common primers used in the literature for the quantification of ARGs, highlighting the necessity to improve the molecular tools currently available for qPCR quantification in order to increase sensitivity and specificity of the assays.

Table 2. Sequences of the primers designed in this study and their major features.

Gene Target	Primer ID	Sequence (5´- 3´)	Primer length (pb)	Amplicon size (pb)
aadA	aadA-336F	CATTCTTGCRGGTATCTTCGAGC	23	215
	aadA-550R	GCACTACATTYCGCTCATCGC	21	
aadB	aadB-118F	GACACAACGCAGGTCACATT	20	419
	aadB-536R	GGTGGTACTTCATCGGCATAG	21	
ampC	ampC-535F	GTGAAGCCRTCTGGTTTGAG	20	494
	ampC-1028R	GCGACATAGCTACCAAATCCG	21	
bla_{SHV}	blaSHV-286F	CAGGATCTGGTGGACTAYTC	20	219
	blaSHV-504R	CGCCTCATTSAGTTCCGTTTC	21	
bla_{TEM}	blaTEM-335F	CGGATGGCATGACAGTAAGAG	21	275
	blaTEM-609R	TTGCCGGGAAGCTAGAGTAAG	21	
*dfrA*1	dfrA-127F	GTMGGSCGCAAGACDTTYGA	20	255
	dfrA-381R	GWARACATCACCYTCTGGCT	20	
ermB	ermB-F	GGAACAGGTAAAGGGCAT	18	434
	ermB-R	TCTGTGGTATGGCGGGTAAG	20	
fosA	fosA-7F	ACCGGTCTCAATCACCTGAC	20	300
	fosA-306R	GAGGAAGTAGAACGAATCGCC	21	
mecA	mecA-1196F	CTTCACCAGGTTCAACTCA	19	370
	mecA-1565R	CCTTGTCCRTAACCTGAATC	20	
qnrS	qnrS-F	GCCAATTGYTACGGKATWGAG	21	227
	qnrS-R	GACTCTTTCARTGATGCRCC	20	
tetA(A)	tetA(A)-F	TCATGCARCTYGTAGGMCAGG	21	454
	tetA(A)-R	AKCCATGCCMAWCCGTTCCA	20	

3.2 Optimal PCR thermocycling profiles for the amplification of ARGs using the new primer sets

The specificity of the new primer sets was tested *in vitro* using as templates genomic DNA of the corresponding reference strains previously described as carrying ARGs in their genomes, as well as environmental DNA pools. After validating different temperatures and times for each of the reaction steps, the thermal profiles that generated better amplification efficiencies are described in Table 3. In all cases, a unique amplification band with the expected amplicon size was observed from both pure cultures (reference strains) and environmental DNA, highlighting the specificity of the molecular tools developed here (Bustin and Nolan, 2017). An example of the *in vitro* validation of the specificity of the new primer sets and PCR amplification efficiency is shown in Figure. 1SI.

Table 3. Thermocycler conditions for quantification of the different ARGs by qPCR.

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

3.3 Validation of Real-Time Quantitative PCR assays

The quantification of the gene copy numbers by qPCR is based on the linear relationship between the logarithm of the initial template quantity and the quantification cycle (*Cq*) value during amplification. A wide linear dynamic range is one of the key performance parameters to achieve in the design of a new qPCR method. This is one of the most important advantages of qPCR assays, since it ensures the accurate quantification of the copy numbers of the target genes, even if the range of abundance spans several logarithmic units (Bustin and Huggett, 2017). Also, the linearity in the qPCR quantification must be reported through the coefficient of determination (R^2 value) between the *Cq* values and the logarithm of gene copy numbers (Xu et al., 2019). In this respect, the standard curves of the *Cq* values and the gene abundances here reported showed good linearity in the range from 20 to 2×10^8 gene copies (see details in Supplementary Figure 2SI), with R^2 values >0.990 (Table 4). Hence, the newly developed qPCR protocols provided proper fits of the distribution of the data and the accurate estimation of the abundances of the different ARGs within a broad linear dynamic range.

Table 4. Equation of the linear regression of standard curves, R^2 value, and amplification efficiency of each qPCR assay.

Target gene	Standard curve	R^2	Amplification efficiency
aadA	*y = -3.507x + 34.893*	0.997	100 %
aadB	*y = -3.4644x + 31.965*	0.995	94 %
ampC	*y = -3.5201x + 33.994*	0.995	92 %
bla_{TEM}	*y = -3.5512x + 35.579*	0.998	91 %
bla_{SHV}	*y = -3.5431x + 34.665*	0.998	92 %
*dfrA*1	*y = -3.5276x + 33.713*	0.998	92 %
ermB	*y = -3.5199x + 33.689*	0.994	92 %
fosA	*y = -3.487x + 33.185*	0.997	94 %
mecA	*y = -3.5821x + 34.339*	0.997	90 %
qnrS	*y = -3.5798x + 34.293*	0.998	90 %
tetA(A)	*y = -3.587x + 35.124*	0.996	90 %

Considering that the abundance of ARGs in certain types of samples may be very low, the development of a qPCR assay must be designed to differentiate a low number of copies of a given ARG in a sample from the inherent noise of the method. In this regard, the *Cq* values of the non-template assays (negative controls) were null or at least 3.3 cycles higher than those of the last dilution of the last standard point; accordingly, no

significant background noise was found in these experiments (Forootan et al., 2017). Besides, it is necessary to calculate the limits of detection (LOD) and quantification (LOQ) of the new qPCR methods as indicators of the quantification accuracy (Forootan et al., 2017). The LOD values, based on detecting the target sequence at the lowest concentration of the standard curve, ranged from 80-120 copy numbers, and similarly, the LOQ values of the assays corresponded to 4×10^4 gene copies per gram of environmental sample, which reflects the assay's capacity to precisely quantify the target genes expressed as gene copies abundance per gram of matrix.

The efficiency values of the different qPCR assays described here ranged between 90 and 100% (Table 4); therefore, good performances of the developed methods were found, according to Keenum et al. (2022). In this respect, variable amplification efficiencies have been reported in the quantification range for several ARGs, including *bla*$_{\text{CTX-M}}$ (95.3%), *bla*$_{\text{TEM}}$ (107.4%), *bla*$_{\text{OXA1}}$ (92.1%), *ermB* (91.3%), *tetA* (95.4%), *sul*1 (95.8%), *sul*2 (83%), *dfrA*1(88.5%), and *dfrA*12 (99.4%) (Xu et al., 2019). The repeatability and reproducibility were determined by comparing the *Cq* values among analytical replicates in a given qPCR assay and those obtained in the different qPCR assays. In this regard, the intra-analytical deviations of the standards among all qPCR assays were very low (average *Cq* value=0.345, ranging from 1.164 to 0.024 cycles). Similarly, the mean analytical deviations among different qPCR experiments were 1.01 cycles (ranging from 0.558 to 1.316).

Finally, the specificity of the qPCR methods was also determined by analyzing the amplicon products after performing the assay (Pochel et al., 2003). In all cases, the melt curves displayed a single sharp peak and were shoulderless (except for the *ermB* gene which specificity was confirmed by Sanger sequencing), indicating that the amplicons obtained were free of unspecific products, highlighting the reliable amplification of the target ARGs (Figure. 3SI). Also, the verification of the melting temperature of the amplicons from environmental samples was compared with the expected peak of melting temperature obtained for the amplification products of the standards. Both melting temperature peaks were equivalent, confirming the specificity of primer annealing previously observed in the assays for the optimization of the PCR conditions. Therefore, these qPCR methods showed a proper level of analytical repeatability and reproducibility, confirming their effectiveness to quantify several ARGs reliably and accurately.

3.4 Total quantification of ARGs in environmental samples

Developing new qPCR methods requires detecting and quantifying the actual occurrence of a given population in complex DNA from different environmental samples, including rare, cryptic, and elusive genes (Beng and Corlett, 2020). For that purpose, the efficiency of the primers, the qPCR conditions, and the effects of different matrices were validated in six natural and engineered environmental samples to show the right level of compliance with the critical considerations that need to be addressed for the validation of the design of new primers and the establishment of newly developed qPCR approaches. The total abundance of the ARGs detected in this study is presented in Figure. 2. The genes *aadA*, *aadB*, *bla*$_{TEM}$, *dfrA*1, and *fos*A were prevalent in all samples. On the other hand, the *ampC*, *bla*$_{SHV}$, *ermB*, *qnrS*, and *tetA*(A) genes were only detected in some samples, suggesting that these ARGs are rare in the different environments analyzed. Finally, the *mecA* gene was not measurable in any samples. According to these results, high ARGs' detection frequencies (ranging from 70 to 100%) were found in the environmental samples using the de novo primer sets and qPCR methodologies proposed in this research, except for the *mecA* gene. The widespread occurrences and ubiquitous distribution of ARGs for the most used antibiotics (beta-lactams, fluoroquinolones, tetracyclines, macrolides, and sulfonamides) in natural and engineered ecosystems have been identified as a severe public health concern (Schages et al., 2021), a fact widely attributed to the subminimum concentrations of antibiotics that reach these ecosystems (Oberoi et al., 2019).

The total abundances of ARGs among all the samples oscillated from 10^3 to 10^8 copies/g of environmental samples. Generally considered, the most abundant ARG was the *aadA* gene (average value 2.81× 10^7 copies/g), followed by *ermB* (2.75 × 10^6 copies/g), *dfrA*1 (2.75 × 10^6 copies/g), *ampC* (1.90 × 10^6 copies/g), *aadB* (1.18 × 10^6 copies/g), *qnrS* (8.10 × 10^5 copies/g), *bla*$_{TEM}$ (5.45 × 10^5 copies/g), $_{tetA}$(A) (3.65 × 10^5 copies/g), *bla*$_{SHV}$ (1.33× 10^5 copies/g), and, finally, *fosA* (1.35 × 10^4 copies/g). According to the Kruskal–Wallis and Conover-Iman tests (Figure 2), *ermB* and *aadA* were the most abundant genes in all the samples in which these ARGs were detected. The abundances of *aadB*, *bla*$_{TEM}$, dfrA1, and *tetA*(A) genes presented a middle prevalence, and the lowest abundances were statistically found for *bla*$_{SHV}$, *fosA*, and *qnrS*. Finally, the numbers of gene copies of *ampC* were highly variable among samples.

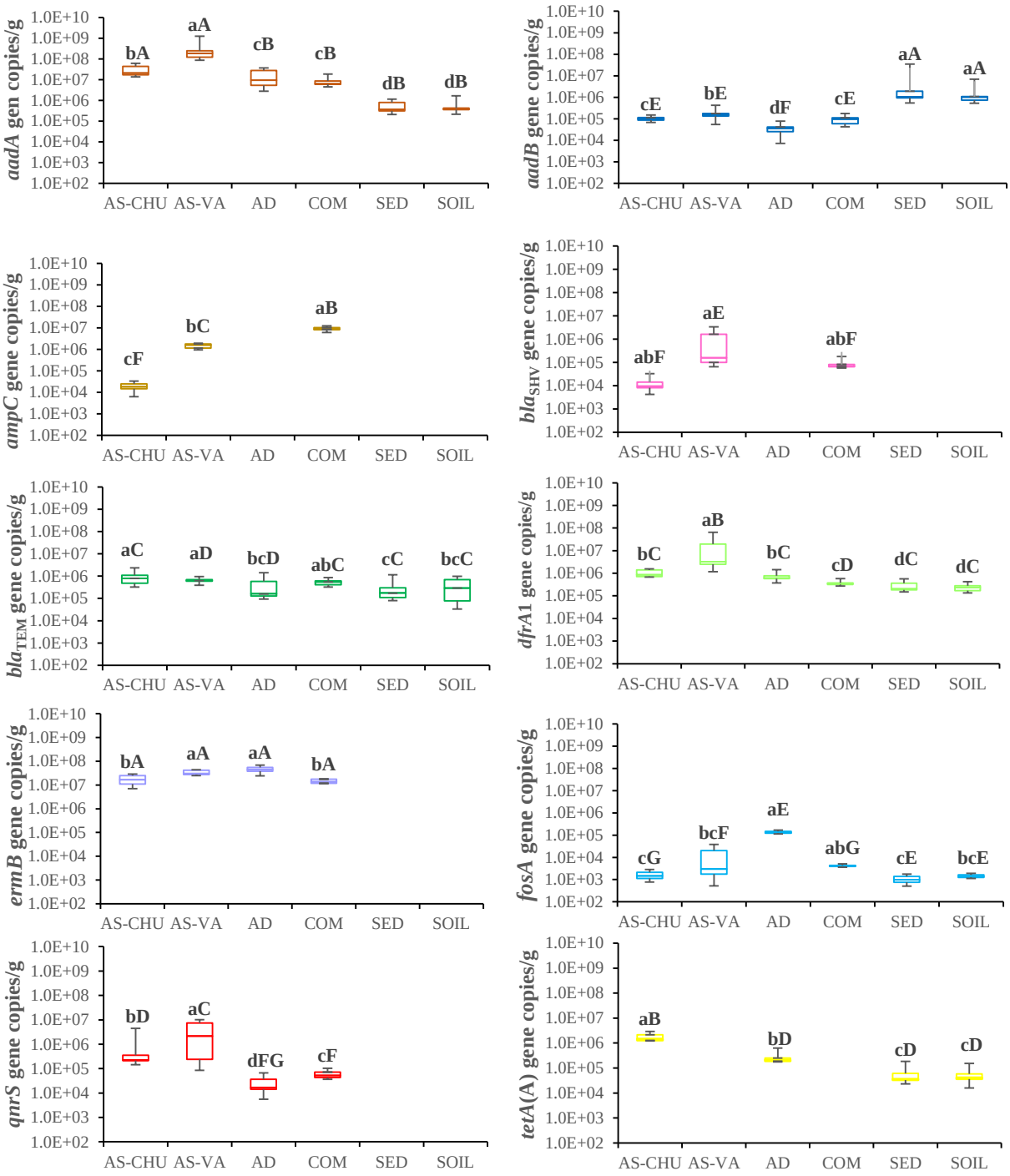

Figure 2. Total abundance of ARGs in the six environmental samples determined as gene copies/g of biomass in two independent qPCR reactions (n=18). According to the Kruskal-Wallis and Conover-Iman tests ($p < 0.05$), different lowercase letters indicate significant differences among environmental samples for a given ARG, and different capital letters indicate significant differences among ARGs for a given environmental sample.

3.5 Relative abundance of ARGs in environmental samples

Figure 3 and Table 4SI display the normalized ratio of ARGs copies to 16S rRNA copies (see details of 16S rRNA values in Figure 4SI). The highest relative abundance of ARGs for aminoglycosides (*aadA*, average 3.02%) and macrolides (*ermB*, 1.00%) observed in this study is in agreement with those previously found in different WWTPs, anaerobic digestates, livestock manure, and riverine ecosystems (Chen et al., 2007; Marti et al., 2013b; Tang et al., 2017). The high prevalence of the *aadA* gene in the six samples regardless of their origin could be linked to the fact that streptomycin has been amply used in animal husbandry and plant disease control since the late 1950s (Wang et al., 2018b), which could have had a strong impact in the development and dissemination of aminoglycoside resistance in the natural environment. However, the use of streptomycin as a first-line antibiotic for tuberculosis treatment makes mandatory to avoid the dissemination of the *aadA* gene within pathogens and other sensitive bacteria (Cohen et al., 2020). Similarly, the *ermB* gene is one of the most abundant antibiotic-resistant genes within the bacterial communities of WW (Wang et al., 2018d), whose dissemination is linked to bacteriophages via transduction, a prevalence that has been connected to the common clinical use of macrolides (Tang et al., 2017).

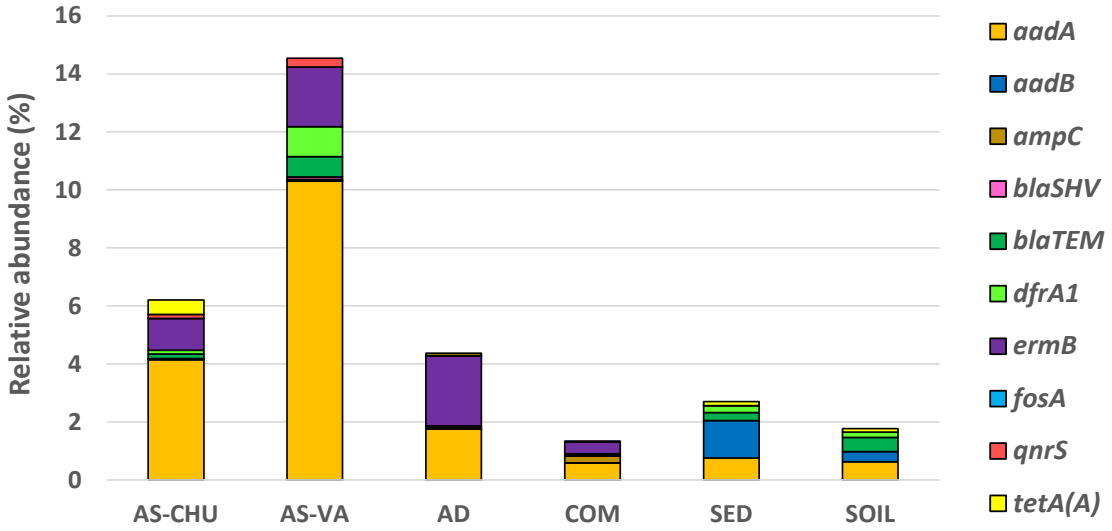

Figure 3. Relative abundance of different ARGs (copies ARG/copies bacterial 16S rRNA) in the six environmental samples determined by qPCR (n=18).

The low relative abundances of *aadB*, *ampC*, *bla*$_{SHV}$, *bla*$_{TEM}$, *dfrA*1, *qnrS*, and *tetA*(A) genes suggest that these ARGs are not the dominant resistance mechanisms in the bacterial communities here analyzed. Similarly, low relative abundances of these genes

have been described in activated sludge from different WWTPs, anaerobic digestate, compost, riverine sediments, and agricultural soil samples for the *aadB* (Tang et al., 2017: Liao et al., 2019a), *ampC* (Wang et al., 2020; Lin et al., 2021), *bla*$_{SHV}$ (Graham et al., 2016; Wang et al., 2020), *bla*$_{TEM}$ (Eramo et al., 2016; Wang et al., 2020; Shin et al., 2022), *dfrA*1 (Mahbub et al., 2020; Keenum et al., 2022; Shin et al., 2022), *qnrS* (Huang et al., 2019; Slipko et al., 2021), and *tetA*(A) genes (Mahbub et al., 2020; Lin et al., 2021). However, although these ARGs were detected in low relative abundances, their common occurrence implies a loss of the therapeutic effects of some of the most employed antibiotics, resulting in treatment failures in both developed and developing countries, and could be important agents in the exacerbation of antibiotic resistance emergence. Ultimately, although all these genes were detected in low relative abundances in the samples analyzed here, monitoring their occurrence in the environment is essential to prevent their overdispersion, which will increase antibiotic resistance emergence irretrievably.

Finally, low relative abundances of *fosA* and the null presence of *mecA* genes were found in the six environmental samples here analyzed. Although only a few studies have determined the occurrence of the *fosA* gene in environmental samples, its scarce detection is in agreement with the previous results found in isolated bacteria (Tuo et al., 2018) and in different environmental samples (Chen et al., 2021). The prevalence of *fosA* in the environment supposes the proliferation of its main reservoirs, which are the well-recognized human pathogens *Klebsiella* spp., *Enterobacter* spp., and *Serratia marcescens* (Ito et al., 2017). Besides, the absence of *mecA* gene in the different environmental samples is in agreement with the previous report of Shoaib et al. (2020). However, a starting high prevalence of methicillin-resistant bacteria in environmental samples, mainly WWTPs, has been addressed in recent years (Pimenta et al., 2023). Therefore, monitoring of both ARGs needs to be conducted and included in the framework for surveillance in natural environments to fill the gaps in the knowledge of the prevalence in environmental samples of *fosA* and *mecA* encoding resistance to therapeutic compounds essential to fight bacterial infections.

4. CONCLUSIONS

In this study, eleven new primer sets targeting *aadA*, *aadB*, *ampC*, *bla*$_{SHV}$, *bla*$_{TEM}$, *dfrA*1, *ermB*, *fosA*, *mecA*, *qnrS*, and *tetA*(A) genes were designed *in silico* fulfilling the strict

requisites recommended for the proper development of primers aimed for qPCR, providing improved designs and higher coverages compared to most currently available primers. The validation of the new primer sets and qPCR protocols showed target specificity and high PCR efficiency, ample linear dynamic range, analytical sensitivity with low LOD and LOQ values, repeatability, and reproducibility of the assays, demonstrating their robustness even in samples carrying very low target DNA concentrations. The reliability of the new primers and qPCR protocols was also successfully validated in environmental samples, including river sediment, agricultural soil, activated sludge, compost, and anaerobic digestate. The abundance trends of ARGs differed among samples, highlighting the prevalence of *aadA* and *ermB* genes in all of them. Low relative abundances of *aadA*, bla_{SHV}, bla_{TEM}, *dfrA*1, *fos*A, *qnrS*, *tetA*(A) genes and a null-presence of *mecA* gene were found. Particularly, the importance of these ARGs in antibiotic resistance emergence combined with the high occurrence of *aadA* and *ermB* genes confirmed the importance of WWTPs as hotspots of ARB dissemination. The development of effective and reliably new qPCR methods represents an outstanding contribution to quantifying the abundance of the ARGs and can provide information about the occurrence of ARB useful to propose new politics that minimize the emergence of antibiotic resistances. Hence, the improved primer sets developed in this study could be considered valuable tools to accurately monitor the ARGs in the environment as the first step to ameliorate antibiotic resistance phenomena.

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Author Contributions

Lizandra Perez-Bou: formal analysis, investigation, term, formal analysis, writing-review and editing, visualization, and supervision. Alejandro Gonzalez-Martinez: funding acquisition, term, conceptualization, resources, writing-review and editing, and visualization. Juan J. Cabrera: term, conceptualization, resources, writing-review and editing, and visualization. Belen Juarez-Jimenez: term, conceptualization, resources, writing-review and editing, and visualization. Belen Rodelas: funding acquisition, term, conceptualization, resources, formal analysis, writing-review and editing, visualization, and supervision. Jesus Gonzalez-Lopez: funding acquisition, term, conceptualization,

resources, writing-review and editing, and visualization. David Correa-Galeote: formal analysis, investigation, term, conceptualization, resources, formal analysis, writing-review and editing, visualization, and supervision.

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Conflict of Interest

The authors declare no competing interests.

SUPPLEMENTARY MATERIAL

Design and validation of primer sets for the detection and quantification of antibiotic resistance genes in environmental samples by quantitative PCR

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Table 1SI. Culture media composition and incubation conditions for refence strain.

Reference strain	Spain collection	Culture media
Escherichia coli ATCC 35218	CECT 943	NUTRIENT BROTH/AGAR I: Beef extract 5 g Peptone 10 g NaCl 5 g Agar powder (only for solid media) 15 g Distilled water 1 L Adjust pH to 7.2. Growth temperature (in °C): 37 Incubation time: 24h Atmospheric needs: aerobic
Escherichia coli K12	CECT 433	
Klebsiella pneumoniae subsp. pneumoniae ATCC 700603	CECT 7787	NUTRIENT BROTH/AGAR II: Beef extract 1 g Yeast extract 2 g Peptone 5 g NaCl 5 g Agar powder (only for solid media) 15 g Distilled water 1 L Adjust pH to 7.2. Growth temperature (in °C): 37 Incubation time: 24h Atmospheric needs: aerobic
Staphylococcus epidermidis RP62A, ATCC 35984	CECT 4184	
Pseudomonas aeruginosa PAO1	CECT 4122	

Table 2SI. Orthology number of the target antibiotic-resistant genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and the selected sequences of each ARG (orthology grade >70%).

KO00984	***aadA* gene-streptomycin 3"-adenylyltransferase**
Escherichia coli APEC O1 (APEC): APECO1_O1R89	
Aeromonas salmonicida subsp. salmonicida A449: ASA_P4G100	
Salmonella enterica subsp. enterica serovar Newport SL254: SNSL254_p_0144	
Desulfurispirillum indicum: Selin_0013	
Salmonella enterica subsp. enterica serovar Heidelberg CFSAN002069: CFSAN002069_23195	
Escherichia coli UMNK88 (ETEC, porcine): pUMNK88_130	
Acinetobacter baumannii AYE: ABAYE3618	
Pseudomonas aeruginosa NCGM2.S1: NCGM2_1759	
Klebsiella aerogenes EA1509E: CCG28765	
Shigella flexneri 2003036: SFy_1440	
Acinetobacter baumannii BJAB0868: BJAB0868_p0101	
Acinetobacter baumannii TCDC-AB0715: ABTW07_3885	
Acinetobacter baumannii BJAB07104: BJAB07104_p0090	
Acinetobacter baumannii AB0057: AB57_0293	
Acinetobacter baumannii MDR-ZJ06: ABZJ_01289	
Aeromonas hydrophila AL06-06: RY45_02925	
Actinobacillus indolicus: EXH44_00950	
Comamonas kerstersii: B5M06_08275	
Enterobacter hormaechei CAV1176: AB284_00830	
Escherichia coli O44 H18 042 (EAEC): EC042_4093	
Klebsiella pneumoniae subsp. pneumoniae KPNIH24: KPNIH24_27470	
Klebsiella pneumoniae 32192: KU54_01030	
Klebsiella pneumoniae 34618: LI86_28235	
Leclercia sp. LSNIH3: C3F35_26160	
Leclercia sp. LSNIH1: C2U54_25010	
Melaminivora sp. SC2-9: C6568_16815	
Pantoea sp. PSNIH2: PSNIH2_21665	
Pantoea sp. PSNIH1: PSNIH1_20585	
Proteus terrae subsp. cibarius: F9282_19600	
Pseudoxanthomonas suwonensis 11-1: Psesu_2826	
Pusillimonas thiosulfatoxidans: CKA81_11630	
Rheinheimera sp. D18: E0Z06_15160	
Rhizobium pusense IRBG74: BN877_II1271	
Salmonella enterica subsp. enterica serovar Choleraesuis: SCH_051	
Salmonella enterica subsp. enterica serovar Typhimurium U288: STU288_1p00740	
Salmonella enterica subsp. enterica serovar Schwarzengrund: SeSA_B0063	
Synechocystis sp. IPPAS B-1465: C7I86_08920	
Venatorbacter cucullus: GJQ55_09870	
Salmonella enterica subsp. enterica serovar Heidelberg 41578: SEEH1578_00460	
Aeromonas hydrophila MX16A: BOQ57_11180	
Citrobacter sedlakii: JY391_23580	
Escherichia coli O83 H1 NRG 857C (AIEC): NRG857_30102	
Klebsiella grimontii: JJJ10_29165	

KO00984	***aadA* gene-streptomycin 3"-adenylyltransferase (*cont.*)**

Klebsiella oxytoca CAV1374: AB185_02720
Pseudomonas putida HB3267: B479_26984
Klebsiella pneumoniae subsp. pneumoniae KPNIH30: KPNIH30_26930
Aeromonas simiae: FE240_15575
Citrobacter freundii: CFNIH1_0615
Salmonella enterica subsp. enterica serovar Typhimurium T000240: STMDT12_C39240
Shigella flexneri 2002017 (serotype Fxv): SFxv_1143
Shigella flexneri Shi06HN006 (serotype Yv): SFyv_1489
Yokenella regensburgei: HEC60_24125
Citrobacter freundii complex sp. CFNIH3: C2U38_26810
Citrobacter sp. CRE-46: AN232_27685
Citrobacter sp. CFNIH10: C2U53_00295
Enterobacteriaceae bacterium ENNIH2: C2U52_00370
Enterobacter roggenkampii 35734: LI67_024955
Klebsiella oxytoca KONIH1: KONIH1_32185
Klebsiella pneumoniae 30660/NJST258_1: KPNJ1_05669
Klebsiella pneumoniae blaNDM-1: FH42_25795
Klebsiella pneumoniae subsp. pneumoniae KPNIH32: KPNIH32_27930
Klebsiella pneumoniae subsp. pneumoniae MGH 78578 (serotype K52): KPN_pKPN4p07059
Klebsiella pneumoniae subsp. pneumoniae KPNIH29: KPNIH29_26630
Nitrosomonas stercoris: Nstercoris_00876
Providencia huaxiensis: CYG50_16055
Shigella sonnei Ss046: SSON_3896
Acinetobacter baumannii MDR-TJ: ABTJ_01327
Neisseria brasiliensis: GJV52_06020
Aeromonas sp. ASNIH3: C2U39_10275
Pasteurella multocida 36950: Pmu_03420
Shewanella sp. FDAARGOS_354: CEQ32_08445
Paralysiella testudinis: JQU52_07645
Escherichia coli SMS-3-5 (environmental): EcSMS35_A0135
Aeromonas sp. ASNIH5: C2U30_02790
Glutamicibacter protophormiae: JQN66_18130
Klebsiella michiganensis E718: A225_R1p0760
Klebsiella pneumoniae subsp. pneumoniae KPNIH10: KPNIH10_26705
Pseudoxanthomonas mexicana: H4W19_04340
Alcaligenes faecalis ZD02: UZ73_19160
Acinetobacter junii: BVL33_11240
Citrobacter farmeri: CI104_25625
Enterobacter hormaechei subsp. steigerwaltii: LI62_012770
Phytobacter ursingii: AB182_03100
Salmonella enterica subsp. enterica serovar Typhimurium DT104: DT104_38561
Salmonella enterica subsp. enterica serovar Typhimurium 138736: CY43_20060
Simplicispira suum: C6571_18320
Serratia marcescens SM39: SM39_pSMC1_34
Thauera humireducens: AC731_003650
Klebsiella pneumoniae JM45: N559_5186
Klebsiella pneumoniae subsp. pneumoniae HS11286: KPHS_p300690

KO00984	***aadA* gene-streptomycin 3"-adenylyltransferase (*cont.*)**
Klebsiella pneumoniae PMK1: PMK1_ndm00221	
Metakosakonia sp. MRY16-398: MRY16398_p20070	
Pseudomonas aeruginosa NCGM 1900: NCGM1900_3708	
Schlegelella thermodepolymerans: IS481_179	
Klebsiella huaxiensis: DA718_21680	
Vibrio cholerae O1 2012EL-2176: EN18_05755	

K17881	***aadB* gene-aminoglycoside 2''-adenylyltransferase**
Enterobacter cloacae ECNIH2: ECNIH2_24600	
Enterobacter cloacae ECNIH4: ECNIH4_23065	
Enterobacter cloacae ECNIH5: ECNIH5_22890	
Klebsiella pneumoniae subsp. pneumoniae MGH 78578 (serotype K52): KPN_pKPN5p08204	
Klebsiella pneumoniae subsp. pneumoniae KPNIH27: KPNIH27_26410	
Klebsiella oxytoca KONIH1: KONIH1_31180	
Klebsiella oxytoca CAV1374: AB185_01215	
Klebsiella quasipneumoniae: AVR78_27585	
Klebsiella quasivariicola: B8P98_29915	
Citrobacter freundii: CFNIH1_06100	
Citrobacter sp. CFNIH10: C2U53_00275	
Phytobacter ursingii: AB182_00470	
Enterobacteriaceae bacterium ENNIH2: C2U52_00390	
Enterobacteriaceae bacterium ENNIH2: C2U52_01405	
Pasteurella multocida 36950: Pmu_03410	
Pseudoxanthomonas mexicana: H4W19_04355	
Pseudomonas putida HB3267: B479_27254	
Acinetobacter baumannii AYE: ABAYE3622	
Acinetobacter baumannii LAC-4: ABLAC_p200010	
Acinetobacter venetianus: HWI77_18550	
Neisseria brasiliensis: GJV52_06000	

K01467	***ampC gene*-beta-lactamase class C**
Escherichia coli K-12 MG1655: b4150	
Escherichia coli K-12 W3110: JW4111	
Escherichia coli K-12 DH10B:ECDH10B_4345	
Escherichia coli K-12 BW2952: BWG_3865	
Escherichia coli K-12 MDS42:ECMDS42_3592	
Escherichia coli O157 H7EDL933 (EHEC): Z5757	
Escherichia coli O157 H7 Sakai (EHEC):ECs_5131	
Escherichia coli O157 H7EC4115 (EHEC):ECH74115_5668	
Escherichia coli O157 H7 TW14359 (EHEC):ECSP_5252	
Escherichia coli O157 H7 Xuzhou21 (EHEC): CDCO157_4817	
Escherichia coli O111 H- 11128 (EHEC): ECO111_5061	
Escherichia coli O26 H11 11368 (EHEC): ECO26_5319	
Escherichia coli O103 H2 12009 (EHEC): ECO103_4948	
Escherichia coli O145 H28 RM13516 (EHEC):ECRM13516_5195	
Escherichia coli O104 H4 2011C-3493 (EAEC): O3K_22880	
Escherichia coli O104 H4 2009EL-2071 (EAEC): O3O_02505	

K01467	***ampC gene*-beta-lactamase class C**	***(cont.)***

Escherichia coli O104 H4 2009EL-2050 (EAEC): O3M_22785
Escherichia coli 55989 (EAEC):EC55989_4707
Escherichia coli O127 H6E2348/69 (EPEC): E2348C_4478
Escherichia coli O55 H7 CB9615 (EPEC): G2583_4979
Escherichia coli O55 H7 RM12579 (EPEC): ECO55CA74_23880
Escherichia coli O78 H11 K80 H10407 (ETEC):ETEC_4499
Escherichia coli O139 H28E24377A (ETEC): EcE24377A_4709
Escherichia coli UMNK88 (ETEC, porcine): UMNK88_5090
Escherichia coli O6 K15 H31 536 (UPEC):ECP_4396
Escherichia coli NA114 (UPEC):ECNA114_4370
Escherichia coli O25b K100 H4-ST131EC958 (UPEC):EC958_4641
Escherichia coli APEC O1 (APEC): APECO1_2239
Escherichia coli APEC O78 (APEC): APECO78_01705
Escherichia coli O9 HS (commensal):EcHS_A4394
Escherichia coli SMS-3-5 (environmental):EcSMS35_4621
Escherichia coli O152 H28 SE11 (commensal): ECSE_4452
Escherichia coli O8 IAI1 (commensal):ECIAI1_4387
Escherichia coli O81ED1a (commensal):ECED1_4939
Escherichia coli O17 K52 H18 UMN026 (ExPEC):ECUMN_4686
Escherichia coli O7 K1 IAI39 (ExPEC):ECIAI39_4617
Escherichia coli O7 K1 CE10: CE10_4891
Escherichia coli B REL606:ECB_04022
Escherichia coli BL21(DE3): ECD_04022
Escherichia coli BL21(DE3): B21_03984
Escherichia coli BL21-Gold(DE3)pLysS AG:ECBD_3879
Escherichia coli O18 K1 H7 UTI89 (UPEC): UTI89_C4750
Escherichia coli O18 K1 H7 IHE3034 (ExPEC):ECOK1_4664
Escherichia coli O45 K1 H7 S88 (ExPEC):ECS88_4738
Escherichia coli O6 K2 H1 CFT073 (UPEC): c5238
Escherichia coli O44 H18 042 (EAEC):EC042_4626
Escherichia coli O83 H1 NRG 857C (AIEC): NRG857_21120
Escherichia coli O150 H5 SE15 (commensal): ECSF_4040
Escherichia coli ATCC 8739: EcolC_3860
Escherichia coli KO11FL:EKO11_4163
Escherichia coli KO11FL: KO11_01575
Escherichia coli ABU 83972:ECABU_c47090
Escherichia coli DH1:EcDH1_3840
Escherichia coli DH1:ECDH1ME8569_4010
Escherichia coli UM146: UM146_21000
Escherichia coli W:ECW_m4514
Escherichia coli W: WFL_21965
Escherichia coli clone D i14: i14_4746
Escherichia coli clone D i2: i02_4746
Escherichia coli P12b: P12B_c4249
Escherichia coli LF82: LF82_0085
Escherichia coli LY180: LY180_21810
Escherichia coli O18 K1 PMV-1 (ExPEC):ECOPMV1_04613

K01467 ***ampC gene*-beta-lactamase class C** ***(cont.)***

Escherichia coli JJ1886: P423_23130
Escherichia albertii: EAKF1_ch1676
Escherichia marmotae: C1192_19230
Escherichia sp. E4742: FEM44_13415
Shigella flexneri 301 (serotype 2a): SF4308
Shigella flexneri 2457T (serotype 2a):S4573
Shigella flexneri 8401 (serotype 5b): SFV_4309
Shigella flexneri 2002017 (serotype Fxv): SFxv_4697
Shigella flexneri 2003036: SFy_6182
Shigella flexneriShi06HN006 (serotype Yv):SFyv_6254
Shigella flexneri NCTC1 (serotype 2a): NCTC1_04677
ShigellaSonneiSs046: SSON_4336
Shigella boydiiSb227 (serotype 4): SBO_4306
Shigella boydii CDC 3083-94 (serotype 18): SbBS512_E4682
Shigella dysenteriaeSd197: SDY_4394
Shigella dysenteriae 1617 (serotype 1): Asd1617_05767
ShigellaSp. PAMC 28760: A0259_02400
Pseudocitrobacter sp. G163CM: G163CM_31430

K18699 ***bla*$_{SHV}$ gene-beta-lactamase class A SHV**

Enterobacter cloacaeECNIH2:ECNIH2_24885
Enterobacter cloacaeECNIH4:ECNIH4_23090
Enterobacter cloacaeECNIH5:ECNIH5_22870
Enterobacter hormaechei subsp. steigerwaltii: LI62_012635
Enterobacter hormaechei subsp. hoffmanniiECNIH3:ECNIH3_23450
Klebsiella pneumoniae subsp. pneumoniae MGH 78578 (serotypeK52): KPN_01607
Klebsiella pneumoniae subsp. pneumoniae MGH 78578 (serotypeK52): KPN_pKPN4p07050
Klebsiella pneumoniae subsp. pneumoniae NTUH-K2044 (serotypeK1):KP1_2635
Klebsiella pneumoniae subsp. pneumoniae HS11286: KPHS_25220
Klebsiella pneumoniae subsp. pneumoniae 1084 (serotypeK1): A79E_2629
Klebsiella pneumoniae subsp. pneumoniaeKPNIH24:KPNIH24_15650
Klebsiella pneumoniae subsp. pneumoniaeKPNIH24:KPNIH24_27130
Klebsiella pneumoniae subsp. pneumoniaeKPNIH27:KPNIH27_12280
Klebsiella pneumoniae subsp. pneumoniaeKPNIH27:KPNIH27_26680
Klebsiella pneumoniae subsp. pneumoniaeKPNIH27:KPNIH27_27300
Klebsiella pneumoniae subsp. pneumoniaeKPNIH29:KPNIH29_12780
Klebsiella pneumoniae subsp. pneumoniaeKPNIH30:KPNIH30_13070
Klebsiella pneumoniae subsp. pneumoniaeKPNIH31:KPNIH31_12155
Klebsiella pneumoniae subsp. pneumoniaeKPNIH32:KPNIH32_12920
Klebsiella pneumoniae subsp. pneumoniaeKPNIH32:KPNIH32_28045
Klebsiella pneumoniae subsp. pneumoniaeKPNIH10:KPNIH10_12585
Klebsiella pneumoniae subsp. pneumoniaeKPR0928:KPR0928_12605
Klebsiella pneumoniae subsp. pneumoniae ATCC 43816KPPR1: VK055_0891
Klebsiella pneumoniaeKCTC 2242: KPN2242_10850
Klebsiella pneumoniae subsp. rhinoscleromatis SB3432: KPR_2492
Klebsiella pneumoniae JM45: N559_2715
Klebsiella pneumoniae CG43: D364_08175

K18699 *bla*$_{SHV}$ gene-beta-lactamase class A SHV *(cont.)*

Klebsiella pneumoniae 30684/NJST258_2: KPNJ2_02871
Klebsiella pneumoniae PMK1: PMK1_03928
Klebsiella pneumoniae blaNDM-1: FH42_03960
Klebsiella pneumoniae 32192: KU54_013590
Klebsiella pneumoniae 34618: LI86_13525
Klebsiella pneumoniae 34618: LI86_27975
Klebsiella michiganensis E718: A225_R1p0365
Klebsiella oxytoca CAV1374: AB185_01315
Klebsiella quasipneumoniae: AVR78_27200
Klebsiella quasivariicola: B8P98_29925
Klebsiella africana: LGL98_13180
Citrobacter freundii: CFNIH1_25995
Citrobacter sedlakii: JY391_23440
Citrobacter farmeri:CI104_19205
Citrobacter farmeri:CI104_26890
Leclercia sp. LSNIH1: C2U54_26600
Leclercia sp. LSNIH3: C3F35_26215
Phytobacter ursingii: AB182_00590
Enterobacteriaceae bacterium ENNIH2: C2U52_00310
Pantoea sp. PSNIH1: PSNIH1_20630
Pantoea sp. PSNIH2: PSNIH2_21575
Shewanella bicestrii: CF168_21960
Klebsiella pneumoniae subsp. pneumoniae 1084 (serotypeK1): A79E_2629
Klebsiella pneumoniae subsp. pneumoniaeKPR0928:KPR0928_12605
Klebsiella pneumoniae 30684/NJST258_2: KPNJ2_02871
Klebsiella pneumoniae subsp. pneumoniaeKPNIH30:KPNIH30_13070
Klebsiella pneumoniae subsp. pneumoniaeKPNIH31:KPNIH31_12155
Shewanella bicestrii: CF168_21960
Klebsiella pneumoniae CG43: D364_08175
KlebsiellapneumoniaeKCTC 2242: KPN2242_10850
Klebsiella pneumoniae subsp. pneumoniaeKPNIH29:KPNIH29_12780
Citrobacter farmeri: CI104_19205
Citrobacter sedlakii: JY391_23440
EnterobacteriaceaebacteriumENNIH2: C2U52_00310
Enterobacter hormaechei subsp. hoffmanniiECNIH3:ECNIH3_23450
Enterobacter cloacaeECNIH5:ECNIH5_22870
Enterobacter cloacaeECNIH4:ECNIH4_23090
Enterobacter hormaechei subsp. steigerwaltii: LI62_012635
Klebsiella michiganensis E718: A225_R1p0365
Klebsiella pneumoniae PMK1: PMK1_03928
Klebsiella pneumoniae subsp. pneumoniaeKPNIH27:KPNIH27_27300
Klebsiella pneumoniae subsp. pneumoniae ATCC 43816KPPR1: VK055_0891
Citrobacter freundii: CFNIH1_25995
Enterobacter cloacae ECNIH2: ECNIH2_24885
Leclercia sp. LSNIH3: C3F35_26215
Pantoea sp. PSNIH2: PSNIH2_21575
Pantoea sp. PSNIH1: PSNIH1_20630

K18699 *bla*$_{SHV}$ gene-beta-lactamase class A SHV *(cont.)*

Klebsiella pneumoniae subsp. rhinoscleromatis SB3432: KPR_2492
Phytobacter ursingii: AB182_00590
Klebsiella oxytoca CAV1374: AB185_01315
Klebsiella quasipneumoniae: AVR78_27200
Klebsiella quasivariicola: B8P98_29925
Klebsiella africana: LGL98_13180
Klebsiella variicolaKP5-1: A593_22675
Klebsiella variicola DX120E:KR75_20505
Klebsiella variicola DSM 15968: SP68_01480
Klebsiella variicola At-22: Kvar_2736
Klebsiella variicola 342: KPK_2780
Enterobacteriaceae bacterium S05: CUC76_02955
Raoultella planticola: B1209_12385
Klebsiella_pneumoniae_ATCC_700603_1
Klebsiella_pneumoniae_ATCC_700603_2

K18698 *bla*$_{TEM}$ gene-beta-lactamase class A TEM

Escherichia coli O111 H- 11128 (EHEC): ECO111_p1-129
Escherichia coli O104 H4 2011C-3493 (EAEC): O3K_25812
Escherichia coli O104 H4 2009EL-2071 (EAEC): O3O_25195
Escherichia coli O104 H4 2009EL-2050 (EAEC): O3M_00505
Escherichia coli O55 H7 RM12579 (EPEC): ECO55CA74_26309
Escherichia coli O25b K100 H4-ST131 EC958 (UPEC): EC958_A0053
Escherichia coli SMS-3-5 (environmental): EcSMS35_A0130
Escherichia coli O17 K52 H18 UMN026 (ExPEC): ECUMN_4830
Escherichia coli O83 H1 NRG 857C (AIEC): NRG857_30085
Escherichia coli JJ1886: P423_25770
Escherichia fergusonii: EFER_1643
Salmonella enterica subsp. enterica serovar Typhi CT18: HCM1.216
Salmonella enterica subsp. enterica serovar Typhimurium U288: STU288_1p00920
Salmonella enterica subsp. enterica serovar Choleraesuis: SCH_018
Salmonella enterica subsp. enterica serovar Heidelberg SL476: SeHA_C1580
Salmonella enterica subsp. enterica serovar Heidelberg 41578: SEEH1578_00420
Salmonella enterica subsp. enterica serovar Newport USMARC-S3124.1: SN31241_6970
Salmonella enterica subsp. enterica serovar Schwarzengrund: SeSA_B0046
Salmonella enterica subsp. enterica serovar Typhi CT18: HCM1.216
Enterobacter cloacae ECNIH2: ECNIH2_26920
Enterobacter cloacae ECNIH4: ECNIH4_23070
Enterobacter cloacae ECNIH5: ECNIH5_22885
Enterobacter cloacae ECNIH2: ECNIH2_25775
Enterobacter hormaechei subsp. xiangfangensis: LI66_24190
Enterobacter hormaechei subsp. steigerwaltii: LI62_00205
Enterobacter hormaechei subsp. hormaechei: LI64_22450
Enterobacter hormaechei CAV1176: AB284_00210
Enterobacter hormaechei CAV1176: AB284_00580
Enterobacter roggenkampii 35734: LI67_024985
Klebsiella pneumoniae subsp. pneumoniae MGH 78578 (serotype K52): KPN_pKPN4p07062

K18698 *bla*$_{TEM}$ gene-beta-lactamase class A TEM *(cont.)*

Klebsiella pneumoniae subsp. pneumoniae MGH 78578 (serotype K52): KPN_pKPN5p08258
Klebsiella pneumoniae subsp. pneumoniae HS11286: KPHS_p200640
Klebsiella pneumoniae subsp. pneumoniae HS11286: KPHS_p300540
Klebsiella pneumoniae subsp. pneumoniae HS11286: KPHS_p300840
Klebsiella pneumoniae subsp. pneumoniae KPNIH24: KPNIH24_26690
Klebsiella pneumoniae subsp. pneumoniae KPNIH27: KPNIH27_27280
Klebsiella pneumoniae subsp. pneumoniae KPNIH30: KPNIH30_27315
Klebsiella pneumoniae subsp. pneumoniae KPNIH32: KPNIH32_27945
Klebsiella pneumoniae subsp. pneumoniae KPNIH10: KPNIH10_27950
Klebsiella pneumoniae subsp. pneumoniae KPR0928: KPR0928_26155
Klebsiella pneumoniae 30660/NJST258_1: KPNJ1_05666
Klebsiella pneumoniae JM45: N559_5242
Klebsiella pneumoniae 30660/NJST258_1: KPNJ1_05808
Klebsiella pneumoniae blaNDM-1: FH42_25780
Klebsiella pneumoniae blaNDM-1: FH42_26475
Klebsiella pneumoniae 32192: KU54_01195
Klebsiella pneumoniae 34618: LI86_28450
Klebsiella oxytoca CAV1374: AB185_00085
Klebsiella oxytoca CAV1374: AB185_00400
Klebsiella oxytoca CAV1374: AB185_01200
Klebsiella oxytoca CAV1374: AB185_02810
Klebsiella oxytoca CAV1374: AB185_03350
Klebsiella oxytoca CAV1374: AB185_04535
Klebsiella aerogenes EA1509E: CCG28759
Klebsiella huaxiensis: DA718_21570
Citrobacter freundii complex sp. CFNIH3: C2U38_26795
Citrobacter farmeri: CI104_26520
Citrobacter sp. CFNIH10: C2U53_00280
Citrobacter sp. CRE-46: AN232_27700
Citrobacter sp. CRE-46: AN232_30620
Phytobacter ursingii: AB182_00235
Phytobacter ursingii: AB182_03045
Enterobacteriaceae bacterium ENNIH2: C2U52_00385
Providencia rettgeri: RB151_017480
Providencia huaxiensis: CYG50_00600
Providencia huaxiensis: CYG50_01310
Haemophilus influenzae 86-028NP (nontypeable): NTHI2055
Haemophilus influenzae 10810 (serotype b): HIB_01180
Haemophilus influenzae R2866 (nontypeable): R2866_0574
Vibrio cholerae O1 2012EL-2176: EN18_05195
Acinetobacter baumannii AB0057: AB57_06335
Acinetobacter baumannii TCDC-AB0715: ABTW07_3874
Acinetobacter baumannii BJAB0868: BJAB0868_01264
Acinetobacter baumannii AbH12O-A2: LX00_17505
Acinetobacter baumannii AB030: IX87_12750
Acinetobacter baumannii AC29: BL01_19105
Neisseria mucosa FDAARGOS_260: A6J88_09500

K18698 *bla*$_{TEM}$ gene-beta-lactamase class A TEM *(cont.)*

Kingella kingae: KKKWG1_0925
Bacillus subtilis BSn5: BSn5_11680
Bacillus mycoides WSBC 10204: bwei_5861
Bacillus tropicus: FJR70_32840

K18589 *dfrA1 (dhfr)* gene-dihydrofolate reductase (trimethoprim resistance protein)

Escherichia coli O83 H1 NRG 857C (AIEC): NRG857_30100
Klebsiella michiganensis E718: A225_R1p0435
Klebsiella aerogenes EA1509E: CCG28764
Pantoea sp. PSNIH2: PSNIH2_21625
Citrobacter sedlakii: JY391_23585
Alcaligenes ammonioxydans: FE795_09135
Shigella flexneri 2002017 (serotype Fxv): SFxv_4142a
Klebsiella pneumoniae subsp. pneumoniae KPNIH30: KPNIH30_26920
Proteus columbae: F1325_18955
Proteus terrae subsp. cibarius: F9282_19605
Vibrio cholerae O1 El Tor FJ147: IR04_08655
Vibrio cholerae O1 2010EL-1786: Vch1786_I0148
Vibrio cholerae O1 2012EL-2176: EN18_06645
Vibrio cholerae O1 MJ-1236: VCD_003671
Providencia huaxiensis: CYG50_12855
Providencia huaxiensis: CYG50_16065
Acinetobacter baumannii AYE: ABAYE3644
Pandoraea sp. XY-2: DRB87_20550
Glutamicibacter protophormiae: JQN66_18125
Leclercia sp. LSNIH1: C2U54_25015
Rheinheimera sp. D18: E0Z06_15075
Kluyvera ascorbata: KATP_49880
Klebsiella huaxiensis: DA718_21685
Enterobacter cloacae ECNIH2: ECNIH2_23760
Enterobacter cloacae ECNIH4: ECNIH4_23410
Enterobacter cloacae ECNIH5: ECNIH5_23325
Enterobacter hormaechei subsp. hoffmannii ECNIH3: ECNIH3_24070
Enterobacter hormaechei subsp. hoffmannii ECR091: ECR091_23360
Klebsiella pneumoniae subsp. pneumoniae KPNIH27: KPNIH27_25815
Klebsiella pneumoniae subsp. pneumoniae KPNIH29: KPNIH29_26440
Klebsiella pneumoniae subsp. pneumoniae KPNIH31: KPNIH31_26610
Klebsiella pneumoniae subsp. pneumoniae KPNIH32: KPNIH32_28030
Klebsiella pneumoniae 30660/NJST258_1: KPNJ1_05734
Klebsiella pneumoniae blaNDM-1: FH42_26825
Citrobacter freundii: CFNIH1_25090
Yokenella regensburgei: HEC60_24120
Pantoea sp. PSNIH1: PSNIH1_18560
Aeromonas simiae: FE240_15580
Escherichia coli SMS-3-5 (environmental): EcSMS35_A0121
Salmonella enterica subsp. enterica serovar Typhi CT18: HCM1.165c
Citrobacter sp. CFNIH10: C2U53_02605

dfrA1 (dhfr)* gene-dihydrofolate reductase (trimethoprim resistance protein) *(cont.)
Leclercia sp. LSNIH3: C3F35_23540

K00561	***ermB* gene-23S rRNA (adenine-N6)-dimethyltransferase**
Streptococcus suis D12 (serotype 9): SSUD12_1565
Streptococcus suis JS14 (serotype 14): SSUJS14_0695
Streptococcus suis SS12 (serotype 1/2): SSU12_0879
Streptococcus pneumoniae G54 (serotype 19F): SPG_1241
Streptococcus suis BM407 (serotype 2): SSUBM407_0952
Streptococcus oralis: SOR_1880
Streptococcus intermedius C270: SII_0641
Enterococcus faecalis D32: EFD32_pA0006
Enterococcus saigonensis: EsVE80_07090
Enterococcus saigonensis: EsVE80_p1-00130
Enterococcus cecorum: NCTC12421_01166
Vescimonas fastidiosa: MM35RIKEN_22660
Vescimonas coprocola: MM50RIKEN_19590
Clostridioides difficile 630: CD630_20070
Clostridioides difficile 630: CD630_20100
Clostridioides difficile 630: CDIF630_02225
Clostridioides difficile 630: CDIF630_02229
Clostridioides difficile 630Derm: CD630DERM_20072
Veillonella nakazawae: VEIT17_15390
Amedibacterium intestinale JCM 30884: Aargi30884_28740
Intestinibaculum porci: SG0102_07200
Vagococcus xieshaowenii: E4Z98_09655
Jeotgalibaca arthritidis: G7057_06385
Streptococcus pneumoniae CGSP14 (serotype 14): SPCG_0172
Enterococcus faecium DO: HMPREF0351_12804
Staphylococcus pseudintermedius ED99: SPSE_1800
Streptococcus suis SC070731 (serotype 2): NJAUSS_0669
Citrobacter sp. CRE-46: AN232_30680
Macrococcus caseolyticus: MCCL_plsB0042
Enterococcus faecium Aus0085: EFAU085_p3012
Enterococcus cecorum: NCTC12421_01166
Streptococcus pneumoniae 670-6B (serotype 6B): SP670_1166
Streptococcus pneumoniae Hungary19A 6 (serotype 19A): SPH_1420
Streptococcus parasuis: KQ224_08640
Streptococcus gwangjuense: D7D53_04400
Enterococcus avium: EH197_00455
Enterococcus raffinosus: J9537_20525
Vagococcus fluvialis: K5K99_14655
Butyricimonas faecalis: D8S85_06020
Bacillus subtilis subsp. subtilis AG1839: BSUB_10005
Enterococcus faecalis V583: EFA0007
Eggerthella sp. YY7918: EGYY_26800
Amylolactobacillus amylophilus: LA20533_04015
Macrococcus caseolyticus: MCCL_plsB0042

K00561	***ermB* gene-23S rRNA (adenine-N6)-dimethyltransferase**	***(cont.)***

Mammaliicoccus lentus: H3V22_01745
Staphylococcus aureus subsp. aureus SA268 (CA-MRSA): SA268_2515
Streptococcus equinus: A6J79_00310
Streptococcus lutetiensis: KE3_0212
Selenomonas sp. oral taxon 136: AXE86_11765
Streptococcus pseudopneumoniae: SPPN_01485
Eubacterium maltosivorans: CPZ25_016270
Streptococcus pneumoniae TCH8431/19A (serotype 19A): HMPREF0837_12197
Streptococcus cristatus: I872_07900
Streptococcus pneumoniae A026 (serotype 19F): T308_09065
Streptococcus pneumoniae Hungary19A 6 (serotype 19A): SPH_1420
Lactobacillus amylovorus 30SC: LAC30SC_10855
Streptococcus suis D9 (serotype 7): SSUD9_1049
Lactobacillus johnsonii DPC 6026: LJP_0027c

KO21253	***fosA* gene-glutathione S-transferase fosA**

Pseudomonas aeruginosaPAO1:PA1129
Pseudomonas aeruginosaPAO1-VE13: N297_1170
Pseudomonas aeruginosaPAO1-VE2: N296_1170
Pseudomonas aeruginosa UCBPP-PA14:PA14_49780
Pseudomonas aeruginosa LESB58: PLES_41901
Pseudomonas aeruginosa M18:PAM18_3903
Pseudomonas aeruginosa NCGM2.S1: NCGM2_2001
Pseudomonas aeruginosa NCGM 1900: NCGM1900_1466
Pseudomonas aeruginosa DK2:PADK2_20025
Pseudomonas aeruginosa B136-33: G655_19595
Pseudomonas aeruginosa RP73: M062_06155
Pseudomonas aeruginosaPA1:PA1S_20240
Pseudomonas aeruginosaPA1R:PA1R_gp4742
Pseudomonas aeruginosa MTB-1: U769_20109
Pseudomonas aeruginosa LES431: T223_21415
Pseudomonas aeruginosa SCV20265: SCV20265_4295
Pseudomonas aeruginosa YL84: AI22_13710
Pseudomonas aeruginosa c7447m: M802_1166
Pseudomonas aeruginosaPAO581: M801_1170
Sphingomonas echinoides: B18_17335

K02545	***mecA* gene-penicillin-binding protein 2 prime**

Staphylococcus aureus Subsp. aureus TW20 (MRSA):SATW20_00740
Staphylococcus aureus Subsp. aureus LGA251 (MRSA):SARLGA251_00260
Staphylococcus aureus Subsp. aureus CN1 (CA-MRSA):SAKOR_00039
Staphylococcus aureus Subsp. aureusSA40 (CA-MRSA):SA40_0033
Staphylococcus aureus Subsp. aureusSA957 (CA-MRSA):SA957_0040
Staphylococcus aureus Subsp. aureusSA268 (CA-MRSA):SA268_0042
Staphylococcus aureus Subsp. aureus Z172 (MRSA/VISA): SAZ172_0072
Staphylococcus aureus Subsp. aureusST228/10388 (MRSA):SAI1T1_2000270

K02545	***mecA* gene-penicillin-binding protein 2 prime**	***(cont.)***

Staphylococcus aureus Subsp. aureusST228/10497 (MRSA):SAI2T2_1000270
Staphylococcus aureus Subsp. aureusST228/15532 (MRSA):SAI3T3_1000270
Staphylococcus aureus Subsp. aureusST228/16035 (MRSA):SAI4T8_1000270
Staphylococcus aureus Subsp. aureusST228/18412 (MRSA):SAI7S6_1000270
Staphylococcus aureus Subsp. aureusST228/16125 (MRSA):SAI5S5_1000270
Staphylococcus aureus Subsp. aureusST228/18341 (MRSA):SAI6T6_1000270
Staphylococcus aureus Subsp. aureusST228/18583 (MRSA):SAI8T7_1000270
Staphylococcus aureus Subsp. aureus FDAARGOS_5: X998_0034
Staphylococcus aureus 04-02981 (MRSA):SA2981_0039
Staphylococcus aureus 08BA02176 (LA-MRSA): C248_0036
Staphylococcus aureus M1 (MRSA): BN843_630
Staphylococcus aureus CA-347 (MRSA): CA347_39
Staphylococcus epidermidis RP62A (MRSE):SERP2521
Staphylococcus haemolyticus JCSC1435:SH0091
Staphylococcus haemolyticusSh29/312/L2:ShL2_00029
Macrococcus caseolyticus: MCCL_plsB0023
Paenibacillus chitinolyticus: PC41400_15270
Staphylococcus aureus Subsp. aureus MRSA252 (MRSA):SAR0039
Staphylococcus aureus Subsp. aureus COL (MRSA):SACOL0033
Staphylococcus aureus Subsp. aureus USA300_TCH1516 (CA-MRSA): USA300HOU_0031
Staphylococcus aureus Subsp. aureus USA300_FPR3757 (CA-MRSA): SAUSA300_0032
Staphylococcus aureus Subsp. aureus M013 (CA-MRSA): M013TW_0045
Staphylococcus aureus Subsp. aureus JKD6159 (CA-MRSA):SAA6159_00031
Staphylococcus aureus Subsp. aureus JKD6008 (MRSA/VISA):SAA6008_00039
Staphylococcus aureus Subsp. aureus 11819-97 (CA-MRSA): MS7_0033
Staphylococcus aureus Subsp. aureus N315 (MRSA/VSSA):SA0038
Staphylococcus aureus Subsp. aureus Mu50 (MRSA/VISA):SAV0041
Staphylococcus aureus Subsp. aureus Mu3 (MRSA/hetero-VISA): SAHV_0040
Staphylococcus aureus Subsp. aureus MW2 (CA-MRSA): MW0031

K18555	***qnrS* gene-fluoroquinolone resistance protein**

Salmonella sp. SSDFZ69: EOS98_23640
Enterobacter chengduensis: FY206_24725
Klebsiella michiganensis E718: A225_R1p1020
Klebsiella quasivariicola: B8P98_29940
Yokenella regensburgei: HEC60_24205
Vibrio kanaloae: BTD91_17595
Photobacterium ganghwense: FH974_24145
Shewanella aestuarii: HBH39_17605
Nitrincola iocasae: F5I99_19250
Enterobacter hormaechei subsp. xiangfangensis: LI66_23080
Klebsiella pneumoniae blaNDM-1: FH42_25850
Vibrio cyclitrophicus: FAZ90_18555
Vibrio atlanticus: VS_II0063
Salinivibrio sp. YCSC6: E8E00_14635
Salinivibrio costicola: HBA18_14580

K08151	***tetA(A)* gene-MFS transporter, DHA1 family, tetracycline resistance protein**
Edwardsiella anguillarum: ETEE_p1002	
Aeromonassp. ASNIH5: C2U30_02750	
Aeromonas hydrophilaMX16A: BOQ57_11140	
Acinetobacter baumannii AB0057: AB57_06305	
Acinetobacter baumannii AYE: ABAYE3597	
Vibrio cholerae O1MJ-1236: VCD_003738	
Vibrio cholerae O12012EL-2176: EN18_05250	
Salmonella enterica subsp.enterica serovar Heidelberg 41578: SEEH1578_00495	
Salmonella enterica subsp.enterica serovar TyphimuriumU288: STU288_2p00065	
Escherichia coli SMS-3-5(environmental): EcSMS35_A0153(tetA)	
Escherichia coli O25b: K100: H4-ST131EC958(UPEC): EC958_A0129(tetA)	
Escherichia coli O104H42009EL-2050(EAEC): O3M_00390	
Escherichia coli O104H42011C-3493(EAEC): O3K_00380	
Escherichia coli O145H28RM13514(EHEC): ECRM13514_5836	
Acinetobacter baumannii AB0057: AB57_06305	
Acinetobacter baumannii AYE: ABAYE3597	
Thauera aromatica: Tharo_3444	
Comamonas kerstersii: B5M06_08220	
Chitinibacter bivalviorum: HQ393_16915	
Alcaligenes ammonioxydans: FE795_09075	
Shewanella bicestrii: CF168_22005	
Shewanella algae: BS332_22230	
Pseudomonas putidaHB3267: B479_26854	
Edwardsiella tardaEIB202: ETAE_p036	
Yokenella regensburgei: HEC60_24170	
Metakosakonia sp.MRY16-398: MRY16398_p20270	
Klebsiella pneumoniae subsp.pneumoniae KPNIH31: KPNIH31_26550	
Salmonella sp. SSDFZ69: EOS98_23680	
Salmonella enterica subsp.enterica serovar Schwarzengrund: SeSA_B0032	
Salmonella enterica subsp.enterica serovar Typhimurium var.5-CFSAN001921: CFSAN001921_23980	
Salmonella enterica subsp. enterica serovar Choleraesuis: SCH_004	
Escherichia coli O83: H1NRG857C(AIEC): NRG857_30067	
Escherichia coli O44: H18042(EAEC): EC042_4111	
Aeromonas allosaccharophila: I6G90_12610	
Aeromonas simiae: FE240_15685	
Shigella sonnei Ss046: SSON_PA02	
Salmonella enterica subsp.enterica serovar NewportSL254: SNSL254_p_0042	
Escherichia coli O152H28SE11(commensal): ECSE_P1-0011	
Escherichia coli APECO1(APEC): APECO1_O1R83(tetA)	
Psychromicrobium lacuslunae: UM93_16920	
Labryssp.KNU-23: FZC33_33810	
Achromobacter xylosoxidansA8: AXYL_03627	
Melaminivora sp.SC2-9: C6568_16750	
Chlamydia trachomatisJ/6276tet1: CTJTET1_04085	
Chlamydia trachomatisRC-J(s)/122: CTRC122_04035	
Chlamydia trachomatisRC-F(s)/852: CTRC852_04130	
Chlamydia trachomatisRC-F(s)/342: CTRC342_04115	

K08151	***tetA(A)* gene-MFS transporter, DHA1 family, tetracycline resistance protein**
Snodgrassella alvi: SALWKB2_0695	
Aeromonas hydrophila AL06-06: RY45_02955	

Table 3SI. Coverages of the newly developed primers and other primers amply used to determine the absolute abundance of different ARGs in the sequences selected from each KEGG orthology number.

Class	Target gene	Primer name	Primer sequence (5´–3´)	Number of target sequences	Percentage of *in silico* amplification	Reference
Aminoglycoside	*aadA*	**aadA-336F**	CATTCTTGCRGGTATCTTCGAGC	96/96	100%	This study
		aadA-550R	GCACTACATTYCGCTCATCGC	96/96	100%	
		aadA-Zhu1F	GTTGTGCACGACGACATCATT	69/96	72%	Zhu et al., 2013
		aadA-Zhu1R	GGCTCGAAGATACCTGCAAGAA	96/96	100%	
		aadA-Zhu-2F	CGAGATTCTCCGCGCTGTA	68/96	71%	Zhu et al., 2013
		aadA-Zhu2R	GCTGCCATTCTCCAAATTGC	92/96	92%	
		aadA-ClarkF	TGATTTGCTGGTTACGGTGAC	73/96	73%	Clark et al., 1999
		aadA-ClarkR	CGCTATGTTCTCTTGCTTTTG	92/96	92%	
		aadA-RandallF	TATCAGAGGTAGTTGGCGTCAT	67/96	67%	Randall et al., 2004
		aadA-RandallR	GTTCCATAGCGTTAAGGTTTCATT	70/96	70%	
		aadA1F	TGAGGGAAGCGGTGATCGCCGAA	67/96	67%	Nugent et al., 2006
		aadA1R	CGCGCTTAGCTGGATAACGCCACG	92/96	92%	
	aadB	**aadB-118F**	GACACAACGCAGGTCACATT	21/21	100%	This study
		aadB-536R	GGTGGTACTTCATCGGCATAG	21/21	100%	
		aadB-RandallF	GAGCGAAATCTGCCGCTCTGG	21/21	100%	Randall et al., 2004
		aadB-RandallR	CTGTTACAACGGACTGGCCGC	21/21	100%	
		aadB-VanhoofF	ATGTTACGCAGCAGGGCAGTCG	21/21	100%	Vanhoof et al., 1992
		aadB-VanhoofR	CGTCAGATCAATATCATCGTGC	21/21	100%	
		aadB-AsadollahiF	ATGGACACAACGCAGGTCGC	21/21	100%	Asadollahi et al., 2012
		aadB-AsadollahiR	TTAGGCCGCATATCGCGACC	21/21	100%	
		aadB-Noppe-LeclercqF	ATCTGCCGCTCTGGAT	21/21	100%	Noppe-Leclercq et al., 1999
		aadB-Noppe-LeclercqR	CGAGCCTGTAGGACT	21/21	100%	
		RH212-R	GCATATCGCGACCTGAAAGC	21/21	100%	Nigro et al., 2011
		RH377-R	GACACAACGCAGGTCACATTGAT	21/21	100%	

Beta-lactams	***ampC***	**ampC-535F**	GTGAAGCCRTCTGGTTTGAG	81/81	100%	This study
		ampC-1028R	GCGACATAGCTACCAAATCCG	81/81	100%	
		ampC-YangF	CCTCTTGCTCCACATTTGCT	55/81	67%	Yang et al., 2012
		ampC-YangR	ACAACGTTTGCTGTGTGACG	80/81	98%	
		ampC-Zhu1F	TGGCGTATCGGGTCAATGT	0/81	0%	Zhu et al., 2013
		ampC-Zhu1R	CTCCACGGGCCAGTTGAG	0/81	0%	
		ampC-Zhu2F	GCAGCACGCCCCGTAA	80/81	98%	Zhu et al., 2013
		ampC-Zhu2R	TGTACCCATGATGCGCGTACT	79/81	96%	
		ampC-Zhu3F	AACAAAAGATCCCCGGTATGG	79/81	96%	Zhu et al., 2013
		ampC-Zhu3R	ACGCCCGTAAATGTTTTGCT	81/81	96%	
		ampC-Zhu4F	CAGCCGCTGATGAAAAAATATG	0/81	99%	Zhu et al., 2013
		ampC-Zhu4R	CAGCGAGCCCACTTCGA	0/81	0%	
		Lak2FP	GGGAATGCTGGATGCACAA	0/81	0%	Volkmann et al., 2004
		Lak1RP	CATGACCCAGTTCGCCATATC	0/81	0%	
		ampC-BöckelmannF	GTGACCAGATACTGGCCACA	0/81	0%	Böckelmann et al., 2009
		ampC-BöckelmannR	TTACTGTAGCGCCTCGAGGA	0/81	0%	
		bl1_ampcF	CCGGATGAGGTCACGGATAC	0/81	0%	Looft et al., 2012
		bl1_ampcR	ATGCTGGCGTTAGCGTAAAGA	0/81	0%	
	blaSHV	**blaSHV-286F**	CAGGATCTGGTGGACTAYTC	89/89	100%	This study
		blaSHV-504R	CGCCTCATTSAGTTCCGTTTC	89/89	100%	
		blaSHV-BuelowF	CTTTCCCATGATGAGCACCT	88/89	99%	Buelow et al., 2017
		blaSHV-BuelowR	AGATCCTGCTGGCGATAGTG	88/89	99%	
		SHV-FW	CGCTTTCCCATGATGAGCACCTTT	88/89	99%	Pei et al., 2006
		SHV-RV	TCCTGCTGGCGATAGTGGATCTTT	79/89	89%	
		blaSHV-Zhu1F	TCCCATGATGAGCACCTTTAAA	88/89	99%	Zhu et al., 2013
		blaSHV-Zhu1R	TTCGTCACCGGCATCCA	84/89	94%	
		blaSHV-Zhu2F	CTTTCCCATGATGAGCACCTTT	88/89	99%	Zhu et al., 2013
		blaSHV-Zhu2R	TCCTGCTGGCGATAGTGGAT	88/89	99%	

	SHV_F	GCGAAAGCCAGCTGTCGGGC	88/89	99%	Henriques et al., 2006
	SHV_R	GATTGGCGGCGCTGTTATCGC	87/89	98%	
	blaSHV-JeminaF	ATTTGTCGCTTCTTTACTCGC	0/89	0%	Jemima et al., 2008
	blaSHV-JeminaR	TTTATGGCGTTACCTTTGACC	0/89	0%	
	blaSHV-RoschanskiF	TCCCATGATGAGCACCTTTAAA	88/89	88%	Roschanski et al., 2014
	blaSHV-RoschanskiR	TCCTGCTGGCGATAGTGGAT	88/89	88%	
	blaTEM-335F	CGGATGGCATGACAGTAAGAG	81/81	100%	This study
	blaTEM-609R	TTGCCGGGAAGCTAGAGTAAG	81/81	100%	
	blaTEM-BuelowF	AAGCCATACCAAACGACGAG	81/81	100%	Buelow et al., 2017
	blaTEM-BuelowF	TTGCCGGGAAGCTAGAGTAA	81/81	100%	
	blaTEM-ZhouF	AGCATCTTACGGATGGCATGA	81/81	100%	Zhu et al., 2013
	blaTEm-ZhouR	TCCTCCGATCGTTGTCAGAAGT	81/81	100%	
	blaTEM-YanF	CATTTTCGTGTCGCCCTTAT	80/81	99%	Yan et al., 2012
	blaTEM-YanR	GGGCGAAAACTCTCAAGGAT	81/81	100%	
	blaTEM-F	TTCCTGTTTTTGCTCACCCAG	81/81	100%	Bibbal et al., 2007
	blaTEM-R	CTCAAGGATCTTACCGCTGTTG	81/81	100%	
blaTEM	**TEM UP**	CACTATTCTCAGAATGACTTGGT	81/81	100%	Lachmayr et al., 2009
	TEM LP	TGCATAATTCTCTTACTGTCATG	81/81	100%	
	TEM_F	AAAGATGCTGAAGATCA	81/81	100%	Speldooren et al., 1998
	TEM_R	TTTGGTATGGCTTCATTC	81/81	100%	
	blaTEM-ToftelandF	ATGAGTATTCAACATTTCCG	81/81	100%	Tofteland eta al., 2007
	blaTEM-ToftelandR	CCAATGCTTAATCAGTGAGG	80/81	99%	
	blaTEM-RoschanskiF	GCATCTTACGGATGGCATGA	81/81	100%	Roschanski et al., 2014
	blaTEM-RoschanskiR	GTCCTCCGATCGTTGTCAGAA	81/81	100%	
	bl2b_tem1F1	TTGACGCCGGGCAAGA	81/81	100%	Looft et al., 2012
	bl2b_tem1R1	TGCTTTTCTGTGACTGGTGAGTACT	81/81	100%	
	bl2b_tem1F2	AGCATCTTACGGATGGCATGA	81/81	100%	Looft et al., 2012

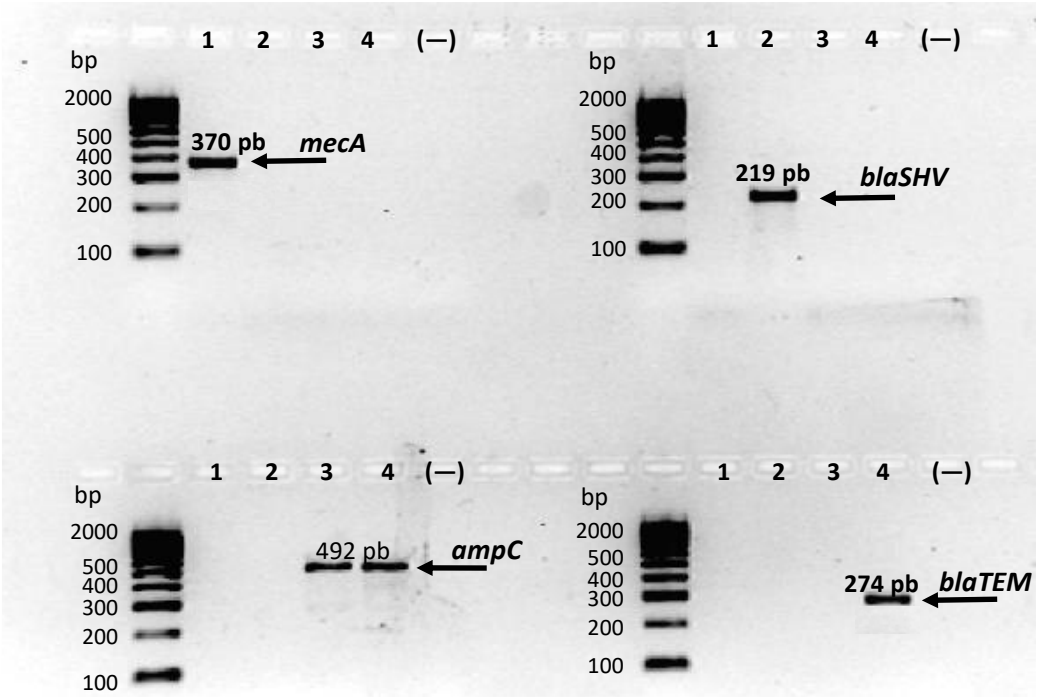
		bl2b_tem1R2	TCCTCCGATCGTTGTCAGAAGT	81/81	100%	
	mecA	**mecA-1196F**	CTTCACCAGGTTCAACTCA	37/37	100%	This study
		mecA-1565R	CCTTGTCCRTAACCTGAATC	37/37	100%	
		mecAF	CATTGATCGCAACGTTCAATTTAAT	35/37	95%	Böckelmann et al., 2009
		mecAR	TGGTCTTTCTGCATTCCTGGA	35/37	95%	
		mecA1FP	CGCAACGTTCAATTTAATTTTGTTAA	35/37	95%	Volkmann et al., 2004
		mecA1RP	TGGTCTTTCTGCATTCCTGGA	35/37	95%	
		mecA-BuelowF	TCCAGGAATGCAGAAAGACC	35/37	95%	Buelow et al., 2017
		mecA-BuelowR	GGCCAATTCCACATTGTTTC	35/37	95%	
Trimethoprim	***dfrA1***	**dfrA1-127F**	GTMGGSCGCAAGACDTTYGA	42/42	100%	This study
		dfrA1-381R	GWARACATCACCYTCTGGCT	42/42	100%	
		dfr1s-f	ATGGAGTGCCAAAGGTGAAC	20/42	48%	Berglund et al., 2014
		dfr1s-r	TATCTCCCCACCACCTGAAA	20/42	48%	
		dfrA1-GrapeF	TGGTAGCTATATCGAAGAATGGAGT	19/42	45%	Grape et al., 2007
		dfrA1-GrapeR	TATGTTAGAGGCGAAGTCTTGGGTA	19/42	45%	
		dfrA1-ZhuF	GGAATGGCCCTGATATTCCA	21/42	50%	Zhu et al., 2013
		dfrA1-ZhuR	AGTCTTGCGTCCAACCAACAG	19/43	45%	
		dfrA1-HoekF	CCAAAGGTGAACAGCTCCTG	19/43	45%	van Hoek et al., 2005
		dfrA1-HoekR	CCAAAGGTGAACAGCTCCTG	19/43	45%	
		dfrA1-GrapeF	ATGGAGTGCCAAAGGTGAAC	19/43	45%	Grape et al., 2007
		dfrA1-GrapeR	TATCTCCCCACCACCTGA AA	19/43	45%	
		dfrA1-RochegüeF	TCGAAGAATGGAGTTATCGGGA	20/43	48%	Rochegüe et al., 2021
		dfrA1-RochegüeR	TGTCAGATGTAAAACTTGAACGTGTTA	19/43	45%	
Macrolides	***ermB***	**ermB-109F**	GGAACAGGTAAAGGGCAT	58/58	100%	This study
		ermB-543R	TCTGTGGTATGGCGGGTAAG	58/58	100%	
		erm(B)-91f	GATACCGTTTACGAAATTGG	58/58	100%	Chen et al., 2007
		erm(B)-454r	GAATCGAGACTTGAGTGTGC	58/58	100%	
		ermB-BuelowF	GGTTGCTCTTGCACACTCAA	58/58	100%	Buelow et al., 2017

		ermB-BuelowR	CTGTGGTATGGCGGGTAAGT	58/58	100%	
		ermB-fw	CGTGCGTCTGACATCTATCTGA	58/58	100%	Mao et al., 2015
		ermB-rv	CTGTGGTATGGCGGGTAAGTT	58/58	100%	
		ermBF	GGATTCTACAAGCGTACCTTGGA	58/58	100%	Böckelmann et al., 2009
		ermBR	GCTGGCAGCTTAAGCAATTGCT	58/58	100%	
		ermB-ZhuF	TAAAGGGCATTTAACGACGAAACT	58/58	100%	Zhu et al., 2013
		ermB-ZhuR	TTTATACCTCTGTTTGTTAGGGAATTGAA	58/58	100%	
		ermB-F	TGAATCGAGACTTGAGTGTGCAA	58/58	100%	Alexander et al., 2015
		ermB-R	GGATTCTACAAGCGTACCTT	58/58	100%	
		ermB-JungF	TTGGATATTCACCGAACACTAGGG	58/58	100%	Jung et al., 2009
		ermB-JungR	ATAGACAATACTTGCTCATAAGTAACGG	58/58	100%	
Fosfomycin	***fosA***	**fosA-7F**	ACCGGTCTCAATCACCTGAC	20/20	100%	This study
		fosA-306R	GAGGAAGTAGAACGAATCGCC	20/20	100%	
		SPI-1559	CACCTCTAGAAGGAGGAAGCCCCCATGCTTACCG GTC	0/20	0%	De Groote et al., 2011
		SPI-1713	CACCGAATTCCTAGTGGTGGTGGTGGTGGTGGTC GGCGAAA CGCATTCCCG	0/20	0%	
		fosA-LiF	ATCTGTGGGTCTGCCTGTCGT	0/20	0%	Li et al., 2015b
		fosA-LiR	ATGCCCGCATAGGGCTTCT	0/20	0%	
		fosA-AbbottF	CGGGTCGAGGAAGTAGAACG	20/20	100%	Abbott et al., 2020
		fosA-AbottR	TGCTCACCGGTCTCAATCAC	20/20	100%	
		fosA-ZhangF	GCTGCACGCCCGCTGGAATA	0/20	0%	Zhang et al., 2020c
		fosA-ZhangR	CGACGCCCCCTCGCTTTTGT	0/20	0%	
Quinolones	***qnrS***	**qnrS-244F**	GCCAATTGYTACGGKATWGAG	15/15	100%	This study
		qnrS-470R	GACTCTTTCARTGATGCRCC	15/15	100%	
		QnrS-F	ATCAAGTGAGTAATCGTATGTACT	10/15	67%	Berglund et al., 2014
		QnrS-R	CACCTCGACTTAAGTCTGAC	10/15	67%	
		qnrS-fw	GTATAGAGTTCCGTGCGTGTGA	7/15	47%	Yan et al., 2012
		qnrS-rv	GGTTCGTTCCTATCCAGCGATT	8/15	53%	

		qnrS-BuelowF	TGGAAACCTACCGTCACACA	2/15	13%	Buelow et al., 2017
		qnrS-BuelowR	AATCGCATCGGATAAAGGTG	2/15	13%	
		qnrSrtF11	GACGTGCTAACTTGCGTGAT	10/15	67%	Marti and Balcázar, 2013
		qnrSrtR11	TGGCATTGTTGGAAACTTG	10/15	67%	
		qnrS UP	CGACGTGCTAACTTGCGTGA	10/15	67%	Colomer- Lluch et al., 2014
		qnrS LP	GGCATTGTTGGAAACTTGCA	10/15	67%	
		qnrS-F	GTGAGTAATCGTATGTACTTTTGC	10/15	67%	Guillard et al., 2011
		qnrS-R	AAACACCTCGACTTAAGTCT	10/15	67%	
		qnrS-F	GCAAGTTCATTGAACAGGGT	10/15	67%	Cattoir et al., 2007
		qnrS-R	TCTAAACCGTCGAGTTCGGCG	10/15	67%	
Tetracyclines	***tetA***	**tetA(A)-737F**	TCATGCARCTYGTAGGMCAGG	49/49	100%	This study
		tetA(A)-1190R	AKCCATGCCMAWCCGTTCCA	49/49	100%	
		tetA-F2-L	CAGCCTCAATTTCCTGACGGGCtG	37/49	76%	Berglund et al., 2014
		tetA-R2	GAAGCGAGCGGGTTGAGA G	37/49	76%	
		tetA-BörjessonF	TCAATTTCCTGACGGGCTG	37/49	76%	Börjesson et al., 2008
		tetA-BörjessonR	GAAGCGAGCGGGTTGAGAG	37/49	76%	
		tetA-NgF	GCTACATCCTGCTTGCCTTC	37/49	76%	Ng et al., 2001
		tetA-NgR	CATAGATCGCCGTGAAGAGG	37/49	76%	
		tetA-F	CAGGCAGGTGGATGAGGAA	37/49	76%	Huang et al., 2015
		tetA-R	GGCAGGCAGAGCAAGTAGAG	37/49	76%	
		tetA-Zhu1F	GCTGTTTGTTCTGCCGGAAA	37/49	76%	Zhu et al., 2013
		tetA-Zhu1R	GGTTAAGTTCCTTGAACGCAAACT	37/49	76%	
		tetA-Zhu2F	CTCACCAGCCTGACCTCGAT	37/49	76%	Zhu et al., 2013
		tetA-Zhu2R	CACGTTGTTATAGAAGCCGCATAG	37/49	76%	
		Tet A-FW	GCGCGATCTGGTTCACTCG	37/49	76%	Aminov et al., 2002
		Tet A-RV	AGTCGACAGYRGCGCCGGC	37/49	76%	
		tetaF	CTCACCAGCCTGACCTCGAT	37/49	76%	Looft et al., 2012
		tetaR	CACGTTGTTATAGAAGCCGCATAG	37/49	76%	

Table 4SI. Relative abundance of different ARGs (copies ARG/copies bacterial 16S rRNA) in the six environmental samples determined by qPCR (n=18).

	AS-CHU	AS-VA	AD	COM	SED	SOIL
aadA	4,149	10,306	1,747	0,589	0,751	0,626
aadB	0,004	0,004	0,001	0,002	1,294	0,345
ampC	0,032	0,054	0,000	0,240	0,000	0,000
bla_{SHV}	0,003	0,077	0,000	0,008	0,000	0,000
bla_{TEM}	0,150	0,706	0,046	0,036	0,276	0,492
*dfrA*1	0,128	1,036	0,068	0,022	0,237	0,186
ermB	1,105	2,050	2,415	0,416	0,000	0,000
fosA	0,000	0,001	0,012	0,000	0,001	0,001
mecA	0,000	0,000	0,000	0,000	0,000	0,000
qnrS	0,133	0,304	0,004	0,005	0,000	0,000
tetA(A)	0,499	0,000	0,071	0,000	0,143	0,121



Carril 1	***Staphylococcus epidermidis* RP62A**
Carril 2	***Klebsiella pneumoniae* ATCC 700603**
Carril 3	***Escherichia coli* K-12 MG1655**
Carril 4	***Escherichia coli* ATCC 35218**
Carril 5	**Control negativo**

Figure 1SI. Examples of *in vivo* validation of the specificity and the efficacy of some of the primers pairs designed in this study by PCR.

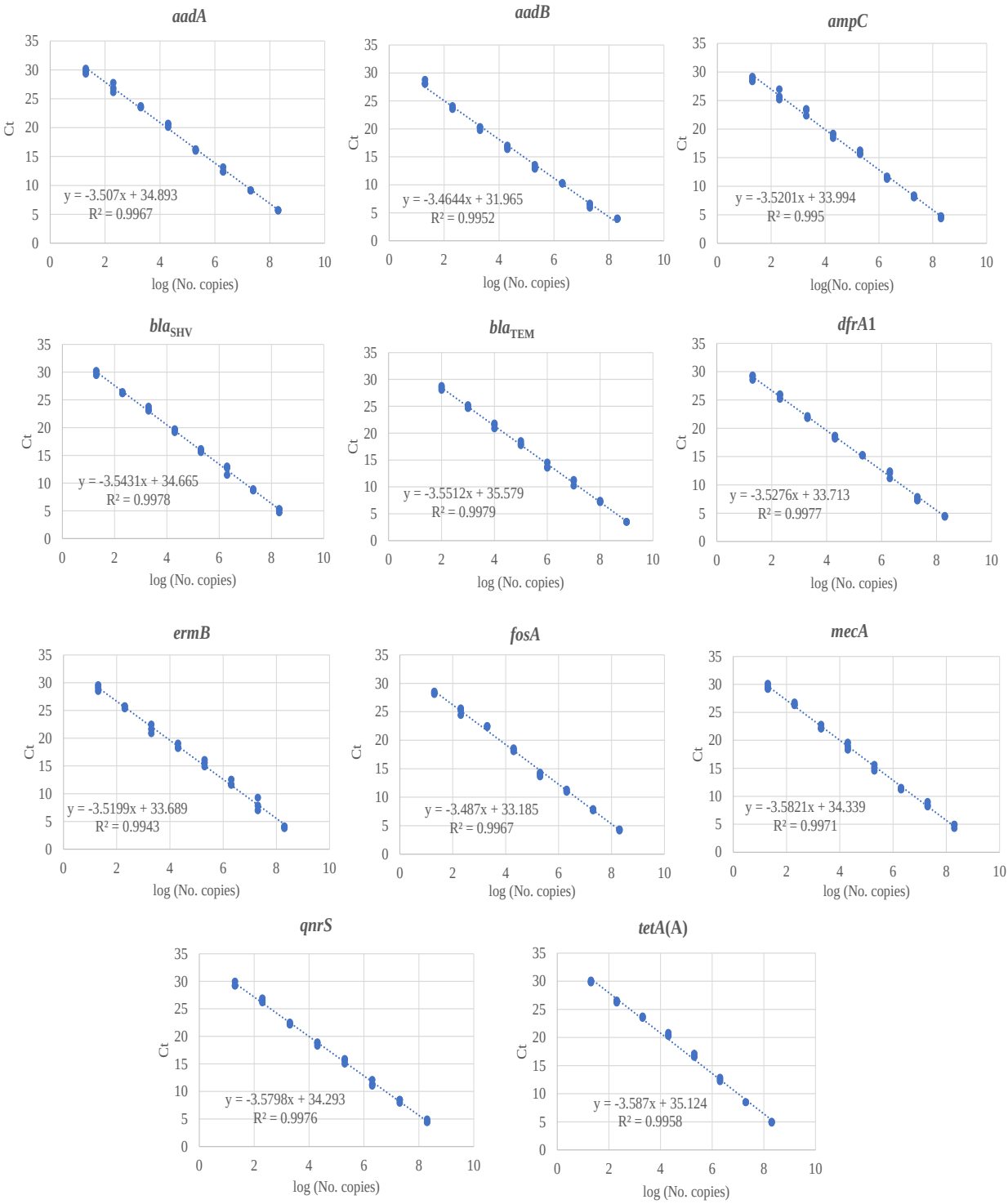


Figure 2SI. Example of standard curves of qPCR for ARGs targets using the new detection primer pairs designed in this study, produced from 10-fold serial dilutions of positive standard plasmid with their curve parameters.

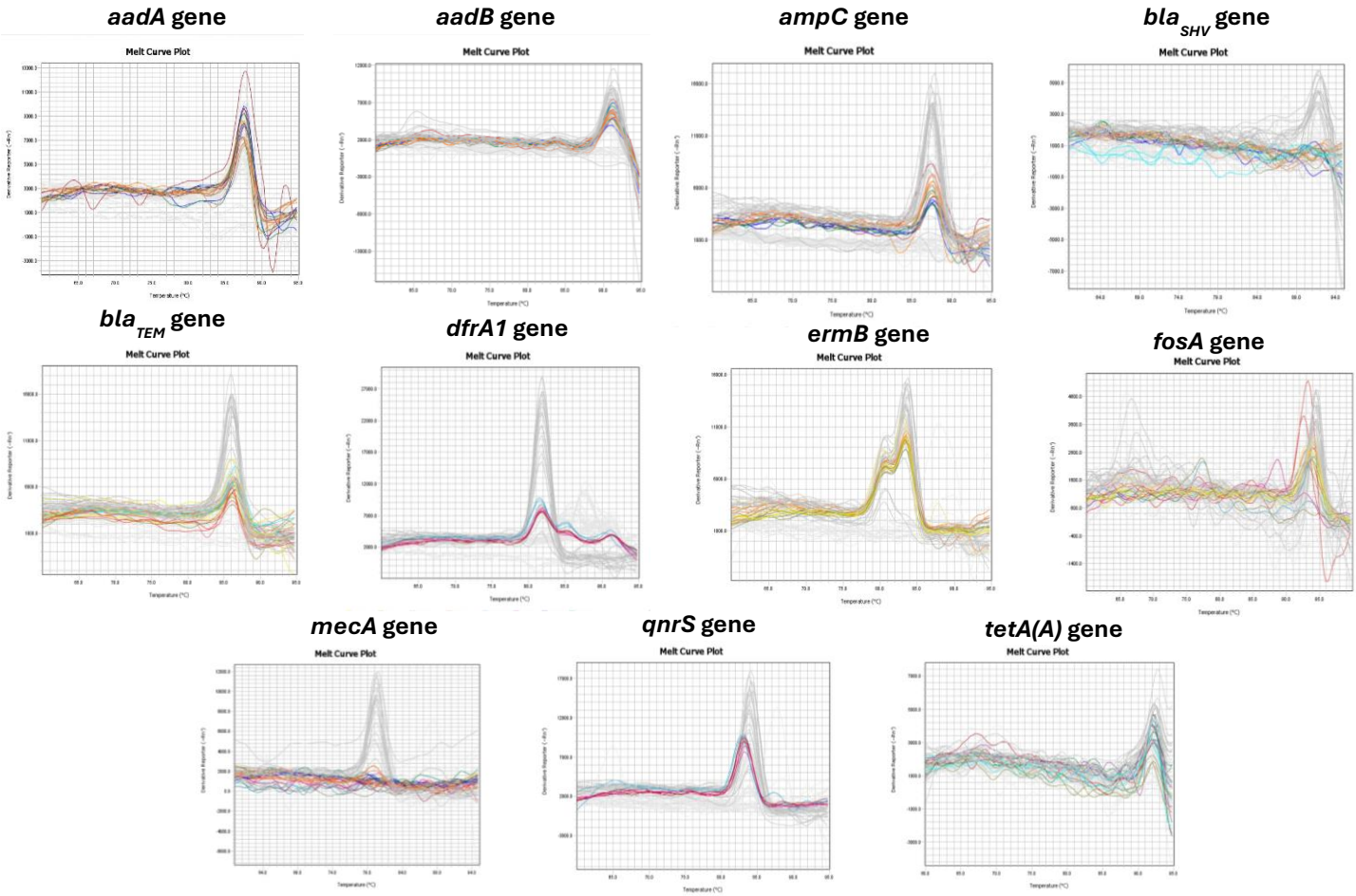


Figure 3SI. Melting curves of qPCR for ARGs targets using the new detection primer pairs designed in this study produced from 10-fold serial dilutions of positive standard plasmid.

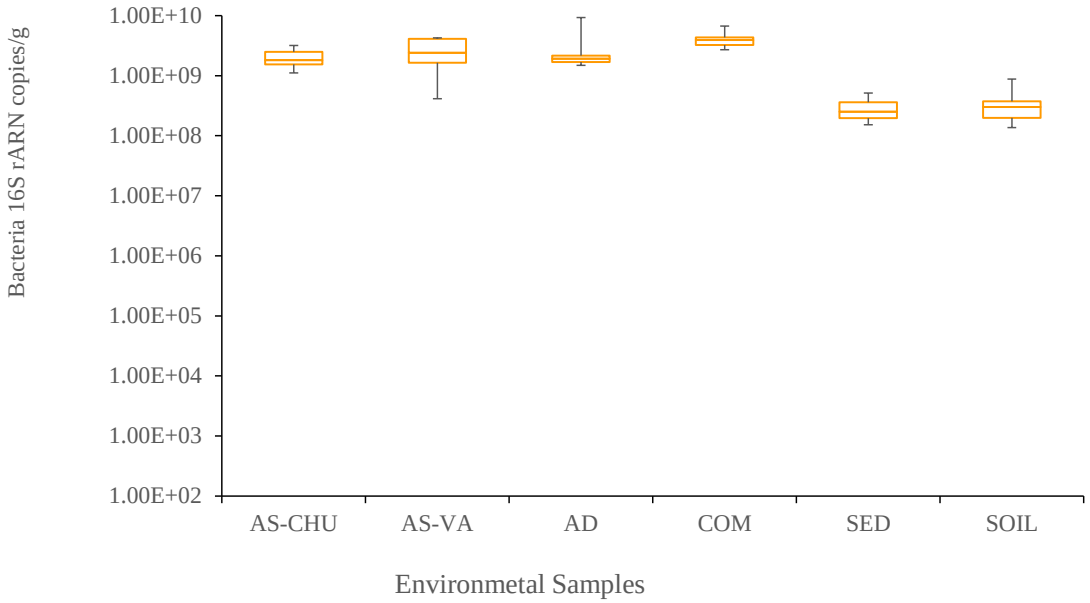


Figure 4SI. Total abundance of the bacterial 16S rRNA gene in the six environmental samples determined as the gene copies/g of biomass in two independent qPCR reactions (n=18).

CHAPTER III

This chapter has been submitted to the Journal of Hazardous Materials in its current form:

Perez-Bou, L., Muñoz-Palazon, B., Carballo Valdes, M.E., Rodelas, B., Gonzalez-Martinez, A., Gonzalez-Lopez; J., Correa-Galeote, D. Dynamics of antibiotic resistance genes under antibiotic occurrence in an aerobic granular sludge system treating real hospital wastewater. Submitted to Journal of Hazardous Materials.

Abstract

Hospital wastewater is one of the most important hotspots for antibiotic resistance dissemination, although this effluent is classified as urban waste directly entering into the municipal sewage system. Treatment of hospital effluents using conventional systems results in a slight reduction of antibiotics, contributing to widespread antimicrobial resistance to the bacterial communities of municipal wastewater plants facilitating the evolution and dissemination of resistomes within different ecosystems. Currently, aerobic granular sludge (AGS) systems are claimed to be the alternative technology to treat complex wastewater; however, the analysis of their performance in treating real hospital wastewater has been extremely limited. Therefore, as an essential tool to significantly reduce the emergence of antibiotic resistance, it is necessary to evaluate the feasibility of aerobic granular technology as an alternative for *in situ* treatment of real hospital effluents prior to discharge into municipal sewer systems to reduce the spread of antibiotic resistance genes (ARGs) into the environment. For that purpose, a laboratory-scale aerobic granular reactor was operated for 150 days, and the concentrations of eleven antibiotic compounds and eleven genes conferring resistance to them were analysed using HPLC/QTOF-MS chromatography and quantitative PCR, respectively. High and variable concentrations of antibiotics were detected in raw wastewater, with ciprofloxacin (7302.8 ng L^{-1}) and sulfamethoxazole (5623.7 ng L^{-1}) being the most abundant. Nevertheless, the system showed great removal rates of over 70% of gentamicin, doxycycline, sulfamethoxazole and ampicillin, although ciprofloxacin and trimethoprim were occasionally released into the effluents. The high exposure to target antibiotics resulted in the accumulation of *aadA*, *aadB*, bla_{TEM}, bla_{SHV}, *ermB*, *fosA* and *qnrS* resistance genes in granular biomass was reinforced, according to the statistically significant positive correlations observed between antibiotic concentrations and the most abundant genes. Hence, the AGS technology is a viable approach to treating hospital wastewater efficiently, with high removal of antibiotics, despite the enrichment of antibiotic resistance genes within the bacterial communities of the granules.

Keywords: antibiotic, antibiotic resistance genes, aerobic granular biomass, hospital wastewater, qPCR

CHAPTER III: Dynamics of antibiotic resistance genes under antibiotic occurrence in an aerobic granular sludge system treating real hospital wastewater

1. INTRODUCTION

Antibiotics are natural or synthetic drugs broadly used to cope with microbial infectious diseases in humans and animals (Puckowski et al., 2016). The therapeutic use of these pharmaceutical active compounds (PhACs) has been hailed as one of the most significant discoveries in human medical history (Friedman et al., 2016; Moser et al., 2019). However, the phenomenon of antimicrobial resistance has been exacerbated by the overuse misuse, or improper use of antibiotics; approximately 65% increase in global antibiotic consumption has increased by over 65% in the last two decades (Klein et al., 2018). In this regard, the negative impact of antimicrobial resistance is reflected by the increase in morbidity and mortality rates of infectious diseases, as well as the high cost of healthcare services (Friedman et al., 2016, Chinemerem et al., 2022). It is estimated that by 2050, antibiotic-resistant infections will claim up to 10 million lives a year and cost the global economy around 100 trillion dollars (Strathdee et al., 2020). Besides, more than 70% of all pathogenic bacteria are thought to be resistant to at least one commercially available antibiotic (Uddin et al., 2021). Hence, the rapid spread of antibiotic-resistant bacteria (ARB) and antibiotic-resistance genes (ARGs) is currently a serious threat to the global environment and human health worldwide (Browne et al., 2021; European Antimicrobial Resistance Collaborators, 2022).

The contributions of hospital wastewater (WW) to the antimicrobial resistance issue have generated special attention due to their significant prevalence of antibiotics, pathogens and ARGs (Hassoun-Kheir et al., 2020; Khan et al., 2021). In addition, their common final disposal into municipal wastewater treatment plants (WWTPs) promotes the proliferation and spreading of antibiotic resistance via ARG dissemination to antibiotic-sensitive bacteria (Hocquet et al., 2016; Hassoun-Kheir et al., 2020; Khan et al., 2021). Accordingly, it is necessary to develop technological solutions to the *in situ* treatment of hospital WW before entering into the general pipelines to reduce the considerable content of antibiotics, ARB and ARGs to achieve municipal WWTPs, ultimately worsening

antibiotic resistance. It is well known that conventional activated sludge (CAS) systems, the most fulfilled WW treatment technology in WWTPs worldwide (Waqas et al., 2020), have been described as an inefficient technology for the removal of emerging pollutants, especially antibiotic compounds (Phoon et al., 2020) and fail to adequately remove primary hotspot of ARGs (Guo et al., 2017). In this manner, members of fluoroquinolones, macrolides, sulfonamides and beta-lactam groups are among the most frequently antibiotics compounds detected in WWTPs (Khan et al., 2021; Langbehn et al., 2021) and their related natural environments (Puckowski et al., 2016). Particularly, it has been amply described the low removal efficiencies of ARGs by CAS treatment of WWTPs receiving hospital waste (Rodriguez-Mozaz et al., 2015; Rafraf et al., 2016).

In recent years, several scientific efforts have been encouraged to improve antibiotic removal (Phoon et al., 2020; Zhu et al., 2021; Khan et al., 2021). In this trend, aerobic granular sludge (AGS) technology has emerged as a promising technology (Nancharaiah et al., 2018), which has been widely deployed from laboratory-scale prototypes (Amorim et al., 2014; Kang et al., 2018a; Muñoz-Palazon et al., 2021 a,b) to full-scale processes (Pronk et al., 2015; Hamza et al., 2022). AGS are typically operated as sequencing batch reactors (SBR) by providing operating conditions that lead to the formation of distinguishable granular biomass compared to that of flocculent sludge in CAS (Nancharaiah et al., 2018; Rosa-Masegosa et al., 2021). In general, WW treatment based on AGS systems is considered reliable, cost-effective and highly efficient and already contributes to the current global WW treatment challenge (Hussain et al., 2024; Perez-Bou et al., 2024a).

Granular biomasses are spherical sludge aggregates of microbial communities through the self-excretion of extracellular polymeric substances (EPS), which provide strong resistance to high loads of toxic compounds and high biomass retention (Kang et al., 2018a; Oliveira et al., 2021; Muñoz-Palazon et al., 2021a). One of the most attractive advantages of this technology is the coexistence of different redox microenvironments within the granule layers, allowing simultaneous removal of carbon, nitrogen, phosphorus and other pollutants in a single reactor (Nancharaiah et al., 2018; Hamza et al., 2022). Regarding PhACs reduction, the metabolically active microbial species of the granular biomass play a critical role in the removal rates through adsorption and degradation mechanisms (Muñoz-Palazon et al., 2021b).

While extensive research has delved into AGS, exploring aspects such as granulation mechanisms, physicochemical performance and diverse applications, mostly using synthetic WW (Amorim et al., 2014; Muñoz-Palazon et al., 2021 a,b; Li et al., 2020 a; He et al., 2021), the ability of AGS to handle real hospital WW with high antibiotics loads properly remains largely unaddressed. In addition, studies on the prevalence of ARGs have predominantly been conducted in traditional flocculent sludge from CAS (Hu et al., 2024), while research on the dynamics and abundance of ARGs under the occurrence of antibiotics in AGS systems are poorly understood.

In this study, an AGS was operated in a laboratory-scale SBR feeding with real hospital WW for 150 days. The AGS system's capacity was mainly explored in terms of removal rates of the most commonly prescribed antibiotics after treatment procedures. The abundance of eleven relevant ARGs (*aadA* and *aadB* - conferring resistance to aminoglycosides); *ampC*, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$ and *mecA* (resistance to beta-lactams); *dfrA*1 (resistance to trimethoprim); *ermB* (resistance to macrolides); *fosA* (resistance to fosfomycin); *qnrS* (resistance to quinolones) and *tetA*(A) (resistance to tetracyclines) were determined in granular biomass samples using recently implemented quantitative PCR (qPCR) protocols. The co-occurrence patterns among ARGs and bacterial abundances during long-time treatment processes were especially addressed. Subsequently, this research contributes to the effective insights into the *in situ* application of AGS technology for treating real hospital WW as an essential approach to reduce the contribution of this highly contaminated WW in terms of antibiotics and ARGs

2. MATERIALS AND METHODS

2.1 AGS reactor operation

A lab-scale AGS reactor (volume 3 L) was operated as SBR during 150 days of operation for treating real hospital WW generated by the University Hospital San Cecilio (Granada, Spain), the biggest hospital in the south of Andalusia. As initial inoculum was employed, 1L of mature granules were previously cultivated at a lab-scale AGS by conventional granulation process. The hydraulic retention time (HRT) was 6.0 h, and the cycles consisted of 4 min for feeding with real hospital water, 170 min of continuous aeration, 3 min of settling, and 3 min for effluent discard, exchanging 50% of total volume per cycle. The air was introduced by fine bubbles at the bottom using a 4 L min^{-1} flow rate. The pH was monitored at 7.6 ± 0.2, and the dissolved oxygen was close to saturation. The

samples of raw influent, effluent water and biomass for analytical procedures were taken on days 0, 7, 15, 30, 45, 60, 90, 120, 140 and 150. Full details regarding the operational parameters of the physicochemical performance and microbial dynamics were previously described by Perez-Bou et al. (2024b).

2.2 Antibiotics quantification in hospital wastewater samples

A total of eleven antimicrobial compounds were analysed in the present study for their detection and quantification (ng L^{-1}) in real hospital WW samples. The following antibiotics compounds were addressed: gentamicin (GEN) and streptomycin (aminoglycoside class); ampicillin (AMP) and amoxicillin (beta-lactams); azithromycin (AZN), clarithromycin (CLM) and erythromycin (ERY) (macrolides); doxycycline (DOX) (tetracycline class); sulfamethoxazole (SMX) and trimethoprim (TMP, trimethoprim-sulfonamides) and ciprofloxacin (CIP) (quinolone class). These compounds were selected according to their broad prevalence and distribution in WWTPs worldwide (Pazda et al., 2019; Mutuku et al., 2022; Perez-Bou et al., 2024a).

A pre-concentration process was necessary to analyse antibiotic compounds to achieve adequate analyte concentrations within the detection limit range of the quantification methods. Extraction columns in solid phase (SPE) Oasis HLB cartridges (200 mg, Milford, CT, USA) were consecutively pre-conditioned with 8 mL of methanol and 8 mL of HPLC water. Then, 100 mL of the collected samples were previously pH adjusted with 0.1 N HCl until they reached pH 4.5 and passed through the same column. To close the pre-concentration process, the cartridges were washed with Milli-Q water (10 mL) and air dried. The extraction process was carried out with a vacuum system adding 2 mL of pure methanol as a solvent to further identify and quantify antibiotics.

Chromatographic analysis was made using a UHPLC-Q-TOF (Agilent LC 1260 Infinity II coupled with a 6470 LC/TQ connected to a Synapt G2S QToF mass spectrometer). The temperature of the column (Columns Zorbax Eclipse plus C18-3 x 50 mm- 1,8 um) was kept constant at 40 ºC. A sample volume of 10 µL was injected, eluting with a mobile positive ionisation phase composed of fractions of H_2O-formic acid 0.1% (A) and acetonitrile (B), and a mobile negative ionisation phase composed of the fractions H_2O-NH_3 0.1% (A) and methanol (B). The gradient sequence was: T 0.10 min = 10% B; T 5.0 min = 90% B; T 7.0 min = 90% B, T 7.10 min = 10% B; T 9.50 min = 10% B with a total analysis time of 9.50 min at a flow rate of 0.40 mL min^{-1}. All antibiotics compounds and

analytical standards were high purity and purchased from Sigma Aldrich (St. Louis, MO, USA ≥ 98% purity). The concentration of the antibiotics was measurable by interpolation in the corresponding standards curves. The linearity in all cases was 99.99%. The range of the standard curves varied from 0.1 to 500 mg mL^{-1}, depending on the antibiotic determined.

2.3 Total DNA extraction

Representative samples (100 mL) of aerobic granular biomass were collected periodically during the aeration step. Then, the biomass was centrifuged at 8000 rpm at 4 ºC for 10 min, and the final pellet was preserved at -80 ºC. Two independent biological replicates (500 mg of granular biomass) were used for DNA extraction by using the FastDNA-2 mL SPIN Kit for Soil and the FastPrep24 apparatus (MP-BIO, CA, USA) according to the manufacturer´s instructions. The quality and concentration of total DNA isolated were verified by visual inspection of electrophoresis on 1% agarose and quantified by spectrophotometry at 260 nm using a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientific, USA). DNA samples were stored at -20 ºC until further use.

2.4 Quantification of total bacteria populations and ARGs abundance by qPCR

The bacterial 16S rRNA genes were amplified using the primers 341F and 534R (Muyzer et al., 1993) to estimate the absolute abundance of bacterial populations as elsewhere described (Correa-Galeote et al., 2021). The prevalence and dynamic of 11 ARGs were analysed according to the developed protocol by Perez-Bou et al. (2024c). In this respect, quantification of ARGs markers, including *aadA* and *aadB* for aminoglycoside resistance; *ampC*, bla_{TEM}, bla_{SHV} and *mecA* for beta-lactam resistance genes analysis; *dfrA*1 as a reference gene to analyse TMP-SMX resistance; *ermB* for macrolide resistance gene; *fosA* coding fosfomycin resistance; *qnrS* to quantify quinolone resistance and *tetA*(A) genes tetracycline resistance genes. Those genes are listed among the ARGs most commonly found in WWTPs (Pazda et al., 2019; Mutuku et al., 2022; Perez-Bou et al., 2024a).

A QuantStudio-3 Real-Time PCR system (Applied Biosystems, USA) equipment was employed to perform the quantitative analysis of genes. Specific determinations (16S rRNA genes and ARGs) were performed as two independent experiments with three independent replicates per DNA sample. The amplification reactions were fulfilled in 25 µL of final volume reaction, according to Correa-Galeote et al. (2021). The quality parameters of high correlation coefficients (R^2 > 0.99) and good efficiencies assays

(ranging between 80% and 105%) were accomplished in all qPCR assays. Standard curves were calculated using ten-fold dilutions of linearised plasmid-carrying inserts of the overmentioned target genes (Correa-Galeote et al., 2013; Perez-Bou et al., 2024c).

2.5 Statistical analysis

Non-parametric statistical tests were employed to determine the significative differences in data by using the Kruskal-Wallis and Conover-Iman combined tests and the Mann-Whitney test ($p < 0.05$ significance level) in XLSTAT v. 2020 (Addinsoft, USA). Furthermore, correlation analysis of the abundance of the different ARGs was made using Spearman's rank correlation coefficients, employing the XLSTAT software.

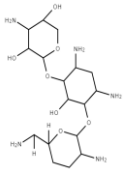
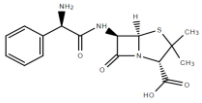
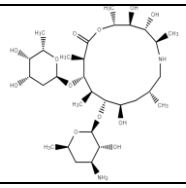
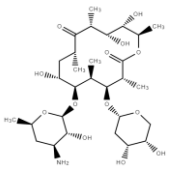
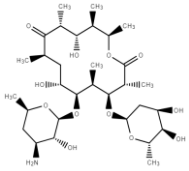
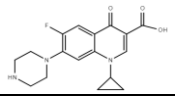
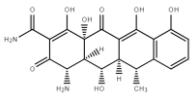
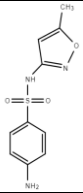
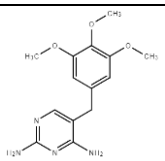
3. RESULTS AND DISCUSSION

3.1 Detection of antibiotics compounds in hospital wastewater

Among all the antibiotics analysed, nine compounds were detected in the hospital WW by the corresponding HPLC/Q-TOF analysis (Table 1); nevertheless, streptomycin and amoxicillin were undetectable throughout the whole experiment. The highest values of concentrations of antibiotics on average in raw hospital WW (n=8) were 7302.8 ± 2704.6 ng L^{-1} and 5623.7 ± 2766.9 ng $L^{-1}$for CIP and SMX, respectively. Notably, these findings are in agreement with the major prevalence of these antibiotics in WW from hospitals and conventional WWTPs and receiving waterbodies worldwide (Omuferen et al., 2022; Matuku et al., 2022).

In this respect, a high presence of CIP has been reported in hospital WW from a district-level of a public hospital in Hanoi, Vietnam (53,300 ng L^{-1}, La Thi et al., 2016), as well as in several multi-specialty hospital centres located in Ujjain, India (236,600 ng L^{-1}, Vishal et al., 2010), Lahore, Pakistan (1800 ng L^{-1}, Ashfaq et al., 2016) and Girona, Spain (13,779 ng L^{-1}, Rodriguez-Mozaz et al., 2015). Similarly, SMX has been detected in significant concentrations in several countries, such as Vietnam (18,900 ng L^{-1}, La Thi et al., 2016), India (81,100 ng L^{-1}, Vishal et al., 2010), Italy (740 ng L^{-1}, Verlicchi et al., 2012) and Spain (4816 ng L^{-1}, Rodriguez-Mozaz et al., 2015).

Table 1. Concentration of the target antibiotics (ng L^{-1}) in hospital WW, molecular structure and relative abundance (%).

Class/Antibiotic	Structure	Concentration (ng L^{-1}) *	Relative abundance (%) **
Aminoglycoside			
Gentamicin		4394.6 ± 2660.3	15.35
Beta-lactam			
Ampicillin		4692.3 ± 1119.3	16.39
Macrolides			
Azithromycin		346.5 ± 112.4	1.21
Clarithromycin		12.6 ± 9.6	0.04
Erythromycin		139.5 ± 60.9	0.48
Quinolones			
Ciprofloxacin		7302.8 ± 2704.6	25.51
Tetracycline			
Doxycycline		1218.1 ± 229.6	4.25
Sulfonamides			
Sulfamethoxazole		5623.7 ± 2766.9	19.64
Pyrimidines			
Trimethoprim		4893.7 ± 490.5	17.09

*Values of concentration in the hospital WW in average (n=8).

** Relative abundance was calculated considering the total concentration of the antibiotics as 100%.

TMP was highlighted as one of the most abundant PhACs detected as it has been noticed as an extremely prevalent antibiotic in surface water from China, Spain, Kenya, and Nigeria and in hospital and urban WWTPs worldwide (Omuferen et al., 2022; Wang et al., 2020). In addition, the common prescription of SMX in combination with TMP, a drug known as co-trimoxazole (Knowles et al., 2020), has contributed to the redistribution of these compounds and their associated ARGs in the environment (Reis et al., 2020).

Beta-lactams and tetracyclines have been reported at moderate levels in hospital WW samples worldwide (Omuferen et al., 2022), data consistent with the levels observed here in the effluents generated by the University Hospital of San Cecilio. Thus, beta-lactam concentrations can vary from 10 to 790 ng L^{-1}ng L^{-1} (Shuaa et al., 2019), 21.2 to 4886 ng L^{-1}, and from 400 to 15, 700 ng L^{-1}, reflecting intermediate levels of prevalence in hospital WW, while tetracycline antibiotics have been detected at values ranging from below 210 ng L^{-1} (Shuaa et al., 2019) to high of 1000 ng L^{-1}. Although tetracyclines are poorly biodegradable due to their complex chemical structure and are very stable in the aquatic environment (Daghrir and Drogui, 2013), DOX concentrations tend to be low in hospital WW, from no detection in effluents in Coimbra, Portugal (Pena et al., 2010) to significant levels in North Italy (970 ng L^{-1}., Verlicchi et al., 2012) and in Kalmar, Sweden (2480 ng L^{-1}, Lindberg et al., 2004), values in agreement with the here found. In contrast, the inherent instability of beta-lactam compounds and their frequent presence as transformation products have resulted in a broad variable occurrence in environmental samples (Zumstein and Helbling, 2019). AMP and amoxicillin have been detected in influent samples at concentrations of 1805 ng L^{-1} and 33,800 ng L^{-1}, respectively, and in effluent at concentrations of 498 ng L^{-1}. and 116,400 ng L^{-1}. (Papageorgiou et al., 2016; Lorenzo et al., 2018; Shuaa et al., 2019). In agreement with the present study, Papageorgiou et al. (2016) indicated a high detection frequency (95.8%) of AMP in contrast to no detection of amoxicillin in municipal WWTPs in Central Greece, as it was found AMP in all the samples in a mean value of 1243 ng L^{-1} and no detection of amoxicillin in any of the samples.

Aminoglycoside and derivate prescriptions have gradually been on the rise in response to the emergence of antibiotic-resistant strains (Pulingam et al., 2022; Zhang et al., 2023) such as re-emerging tuberculosis diseases (Xie et al., 2011). Despite the slight aminoglycoside detection in WW in the last decade (Zhang and Occurrence, 2011), their prevalence has been growing in WWTPs in the last years (Tang et al., 2017; Wang et al.,

2020; Nguyen et al., 2021) due to the contribution of hospital effluents and WW from factories producing these PhACs (Tahrani et al., 2016). In particular, GEN has been found at high concentrations from 5600 (Ebrahimi et al., 2020) to 7600 ng L^{-1} (Löffler and Ternes, 2003) in hospital effluents, with values of prevalence similar than detected here (4394 ng L^{-1}). The macrolides (AZN, CLM, and ERY), these emerging pollutants have been frequently detected in water samples throughout the European region (Omuferen et al., 2022; Mutuku et al., 2022). Nevertheless, AZN (346.5 ng L^{-1}), CLM (12.6 ng L^{-1}) and ERY (139.5 ng L^{-1}) concentrations in hospital WW quantified here were lower than those reported elsewhere (AZN, 425 ng L^{-1}), (CLM, 3976 ng L^{-1}), (ERY, 1587 ng L^{-1}), Omuferen et al., 2022). Although these results are consistent with the moderate or low prevalence of these compounds compared to other groups of antibiotics (Verlicchi et al., 2012; Rodriguez-Mozaz et al., 2015; Ekwanzala et al., 2020), their frequent detection reinforces their persistence in the environment. In addition, it should be stressed the lowest concentration of CLM here detected, although this PhAC is one of the most frequently prescribed macrolides in human medicine (Rodríguez-González et al., 2023), particularly in chronic gastritis and peptic ulcer disease caused by *Helicobacter pylori* in Spain (Mormeneo et al., 2023). Although the types and concentrations of antibiotics in hospital effluents vary according to the hospital category and scale (Guo et al., 2021), the importance of these effluents in the antibiotic contamination of the entire ecosystem should not be neglected, stating the necessity of the *in situ* treatment of the hospital WW before their arrival to the local sewage systems. Also, to implement politics geared to ameliorate antibiotic resistance issues, the inclusion of these antibiotic members in monitoring studies should be conducted.

3.2 Removal of antibiotics compounds in the AGS bioreactor

The concentration of antibiotics in the influent and effluent samples and the removal efficiency expressed as elimination rate (ER %) are presented in Table 2. There is to note, the persistence of AMP, CIP, AZN, DOX, and TMP, as they were present in all sample days. Regarding the AGS's elimination performance, variable ERs were observed among the operational days and antibiotic classes.

Table 2. Removal efficiency (ER %) and antibiotic concentrations (ng L^{-1}ng L^{-1}) in hospital WW effluent in comparison with influent on specific operational days. Values lower than limit of detection (<LoD) were omitted, and ER non determined (n.d) and negative rates were declared.

Antibiotic concentrations (ng L^{-1}) in hospital WW samples										
Day		**GEN**	**AMP**	**AZN**	**ERY**	**CLM**	**CIP**	**DOX**	**SMX**	**TMP**
7	Influent	<LoD	8181.4 ± 9,5	231.8 ± 10,2	<LoD	<LoD	1088.7 ± 43,4	2036.5 ± 51,5	<LoD	2922.2 ± 1,3
	Effluent	<LoD	4349.8 ± 24,9	16.0 ± 3,3	3.6 ± 0,1	1.8 ± 0,1	4406.8 ± 91,3	1363.7 ± 81,4	31.8 ± 4,4	570.9 ± 11,6
	ER %	n. d	46.8	93.1	n. d	n. d	-304,76	33.0	n. d	80.5
15	Influent	15 807.6 ± 575,9	1938.0 ± 1,5	658.3 ± 13,2	251.4 ± 15,1	4.7 ± 0,5	17 451.0 ± 664,6	800.7 ± 9,6	17 514.7 ± 177,7	5734.6 ± 109,6
	Effluent	<LoD	14 735.9 ± 99,5	70.6 ± 5,4	<LoD	<LoD	2586.3 ±106,5	1297.2 ± 65,5	109.9 ± 8,2	3952.5 ± 206,4
	ER %	100	-660,36	89.3	100	100	85.2	-62,01	99.4	31.1
30	Influent	<LoD	3222.0 ± 140,1	24.3 ± 1,0	<LoD	<LoD	535.7 ± 11,9	2349.0 ± 15,4	163.5 ± 6,3	9618.2 ± 33,0
	Effluent	<LoD	2565.2 ± 28,8	10.3 ± 0,5	<LoD	<LoD	1154.8 ± 2,6	198.7 ± 8,1	168.4 ± 2,5	804.3 ± 108,9
	ER %	n. d	20.4	57.5	n.d	n. d	-115,55	91.5	-2,99	91.6
45	Influent	2133.2 ± 86,2	7263.8 ± 40,5	882.7 ± 33,0	474.6 ± 12,0	6.8 ± 0,8	11 682.4 ± 438,6	726.1 ± 34,7	10 207.6 ± 19,6	2435.1± 93,9
	Effluent	<LoD	836.3 ± 34,6	9.5 ± 0,2	<LoD	<LoD	3656.0 ± 46,9	154.8 ± 9,7	34.4 ± 2,3	213.3 ± 1,9
	ER %	100	88.5	98.9	100	100	68.7	78.7	99.7	91.2
60	Influent	<LoD	9693.4 ± 41,9	213.3 ± 0,3	190.4 ± 1,6	5.2 ± 0,4	8140.7 ± 137,5	547.8 ± 18,3	83.9 ± 4,0	12 385.6 ± 11,8
	Effluent	<LoD	4794.2 ± 37,7	21.1 ± 1,3	6.7 ± 0,6	<LoD	2190.1 ± 36,0	156.8 ± 23,6	84.9 ± 6,5	1101.1 ± 30,3
	ER %	n. d	50.5	90.1	96.5	100	73.1	71.4	-1,15	91.1
90	Influent	17 216.4 ± 651,9	1979.6 ± 48,7	579.1 ± 9,3	196.5 ± 12,4	4.3 ± 1,0	18 066.9 ± 175,3	908.7 ± 10,0	16 448.9 ± 54,4	3019.2 ± 66,4
	Effluent	<LoD	1435.4 ± 60,9	21.7 ± 0,2	20.2 ± 6,3	<LoD	4189.0 ± 154,8	100.3 ± 8,8	989.0 ± 21,8	3062.6 ± 131,4
	ER %	100	27.5	96.3	89.7	100	76.8	89.0	94.0	-1,44
120	Influent	<LoD	2018.5 ± 161,4	47.3 ± 4,0	2.8 ± 0,4	<LoD	573.1 ± 6,3	1047.2 ± 26,6	204.0 ± 14,4	2821.6 ± 18,2
	Effluent	<LoD	<LoD	3.8 ± 0,3	<LoD	<LoD	1152.1 ± 22,8	55.0 ± 6,3	55.7 ± 5,9	550.8 ± 57,6
	ER %	n.d	100	92.1	100	n. d	-101,03	94.7	72.7	80.5
150	Influent	<LoD	3241.3 ± 68,0	134.9 ± 8,6	<LoD	79.4 ± 1,3	883.9 ± 19,0	1328.6 ± 14,2	366.8 ± 36,4	213.0 ± 5,3
	Effluent	<LoD	4427.5 ± 56,3	28.8 ± 2,3	<LoD	<LoD	1267.7 ± 26,6	89.0 ± 7,9	41.9 ± 3,8	414.9 ± 9,2
	ER %	n. d	-36,59	78.7	n. d	100	-43,43	93.3	88.6	-94,80

Gentamicin (GEN), ampicillin (AMP), azithromycin (AZN), clarithromycin (CLM), erythromycin (ERY), doxycycline (DOX), sulfamethoxazole (SMX), trimethoprim (TMP) and ciprofloxacin (CIP)

Generally considered, maintained high removal efficiencies (>70%) were observed for all the antibiotics in the sampling days, except for AMP and CIP removal rates, which exhibited great variabilities. For instance, ER in average values for GEN (100%), CLM (100%), ERY (97%), SMX (91%), TMP (90%), AZN (87%), CIP (76%) and DOX (79%); were successfully achieved. The AMP was poorly removed with ER close to 50%. Furthermore, it should be noted that some removal rates were punctually negative.

The main biological mechanism of fluoroquinolones, macrolides, tetracyclines, and TPM removals is via adsorption mechanisms in the AGS technology (Métivier et al., 2013; Xu et al., 2013; Song et al., 2014; Zhang et al., 2018; Muñoz-Palazon et al., 2021b). It is hypothesised that the high content of EPS of the AGS facilitates the adsorption onto the surface biofilm of antibiotics, increasing the removal efficiency of these compounds (Kang et al., 2018a; Nancharaiah et al., 2018; Oberoi et al., 2019). Particularly, the sorption onto the EPS layer in granular biomass has been proposed as the main exclusive removal mechanism of TMP due to its high resistance to elimination via biodegradation (Li and Zhang, 2010).

On the other hand, biodegradation is the main pathway for sulfonamides and beta-lactams degradation (Müller et al. 2013; Zhu et al., 2021), without significant impact on the treatment performance and granular stability (Kang et al., 2018a; Cui et al., 2021). In this regard, sulfonamides biotransformation is highly dependent on nitrogen availability (Müller et al., 2013) and loading organic rate (Liu et al., 2019 b; Cui et al., 2021), by a process specifically carried out by ammonia-oxidising bacteria and sulfate-reducing bacteria (Wang and Wang, 2018). The high removal efficiency of SMX (94%) at a loading dose of 2000 ng L^{-1} in a lab-scale AGS system has been reported (Cui et al., 2021), which is consistent with the 91% achieved in this study. The removal mechanism of AMP is by the readable hydrolysis of the beta-lactam ring, both abiotically and enzymatically, due to its inherent instability in aqueous solutions (Zumstein and Helbling, 2019). In particular, the hydrolysis by microorganisms is the initial step in their degradation facilitating high elimination rates (Hirte et al., 2016; Arsand et al., 2018), which have been notified at different loading doses of AMP in a lab-scale AGS system (Wang et al., 2017b).

The negative elimination rates of some antibiotics detected here (Table 2) could occur due to the reversible nature of adsorption/desorption mechanisms via electrostatic and

hydrophobic interactions (Oberoi et al., 2019; Phoon et al., 2020), as no further sorption could take place when the maximum adsorption capacity of the granules was reached (Ferreira et al., 2016). Then, a potential release of antibiotics to water phase can occur via alternative adsorption/desorption reactions, previous accumulation onto granular surface matter, as they could take place in the AGS operations as has been previously suggested (Amorim et al., 2014; Muñoz-Palazon et al., 2021b; Perez-Bou et al., 2024b), although high removal rates were achieved when the influent and effluent data are analysed together. Specifically, the negative removal rates of CIP in 4 of the 8 sampling days analysed must be shown. Although CIP has a very low Henry's Law constant, defining its high affinity for solid compartments such as sludge, sediments and soils (Puckowski et al., 2016), is also an amphoteric compound promoting their desorption from biosorbent surface conditional to the pH values (Puckowski et al., 2016; Ferreira et al., 2016). In this way, alternative CIP sorption-desorption reactions could occur in the operated AGS. Also, its recalcitrant character and the long half-life time of this molecule could be linked to the scarce removal rates here found, which is consistent with the findings reported by Amorim et al. (2014). Therefore, the high antibiotic ERs shown in this study highlight the ability of AGS technology to significantly reduce the concentrations of antibiotic compounds of real hospital WW after their *in situ* treatment. In particular, this system can enhance the removal of antibiotics by both adsorption and degradation mechanisms. In addition, it is worth noting the good performance of AGS in terms of granular stability, removal of common nutrients (organic matter, nitrogen, and phosphorus) and other PhACs elimination, as previously reported by Perez-Bou et al. (2024b), reinforcing the capabilities of AGS as an alternative to reduce the negative impact of hospital WW in the global antibiotic resistance issue.

3.3 Variation of total abundance of bacteria and ARGs

The values of the gene copies of the bacterial 16S rRNA genes (Figure S1), as a proxy of the bacterial quantification, denoted certain stability in the range of 10^{10} to 10^{11} magnitude orders, which were previously informed by Perez-Bou et al. (2024b). The total abundance of the different ARGs among overall sampling days oscillated from 10^4 gene copies/g of biomass to 10^8 gene copies/g of biomass by the corresponding qPCR analysis (Figure 2). The genes *aadA*, *aadB*, *ampC*, *bla*$_{\text{TEM}}$, *dfrA*1 and *ermB* were detected in all days of operation. However, the occurrence of *bla*$_{\text{SHV}}$, *fosA*, *qnrS* and *tetA*(A) genes were oscillatory with non-detection on certain days. The sporadic detection of genes *bla*$_{\text{SHV}}$,

fosA, *qnrS*, and *tetA*(A) could be mostly attributed to a transient presence of the bacteria-harboring these genetic determinants with poor ability to colonise the preformed granules used in this experiment, which could also be diminished after an intense exposure of the contaminants found in the raw hospital effluents. The most abundant ARGs (in average values) was the *aadA* gene (3.16×10^8 copies/g). The abundances of *aadB* (8.28×10^7 copies/g), *dfrA*1 (1.26×10^7 copies/g), *ermB* (1.94×10^7 copies/g), *qnrS* (1.18×10^7 copies/g) and *tetA*(A) (9.32×10^7 copies/g) had a middle prevalence. Notably, the lowest abundances were found for genetic determinants associated with resistance to the beta-lactam class of antibiotics (*bla*$_{SHV}$: 7.62×10^6 copies/g, *bla*$_{TEM}$: 6.32×10^6 copies/g, *ampC*: 2.00×10^5 copies/g) and to fosfomycin resistance *fosA* gene (4.77×10^4 copies/g). Finally, the *mecA* gene, which confers resistance to all beta-lactam compounds (Liao et al., 2018), was not detected in any samples, even though a high prevalence of this gene in hospital environments is often described (Turner et al., 2019; Shoaib et al., 2020). Hence, different prevalence patterns of the ARGs were broadly evident in the inoculum and granular biomasses in subsequent days of operation.

The abundances of ARGs detected in this research were in the same range as those previously described for different WW treatment bioprocesses, such as CAS (Wei et al., 2018, Perez-Bou et al., 2024 d) and membrane reactors (MBR) (Wen et al., 2018), in the sediments of effluent-receiving areas of WWTPs (Chen et al., 2020), except for the beta-lactam resistance genes. This strikingly low prevalence of *ampC*, *bla*$_{SHV}$, and *bla*$_{TEM}$ are at odds with the evidence of a common high incidence of extended-spectrum beta-lactamase (ESBL) determinants (Raphael et al., 2021). This trend could probably be related to the differences in the epidemiology of ESBL in the different hospital settings, as has been previously suggested by Hocquet et al. (2016) when analysed several hospital WW around the world. These differences in the abundance of ARGs and hosts in each hospital WW suggest the shaping of a unique microbiome according to the type of healthcare service. Besides, raw water could be acting as an indirect influence driver, as well as environmental factors and the structure of bacterial community may also significantly influence the spread and accumulation patterns of ARGs in AGS (Li et al., 2020a).

According to the Kruskal-Wallis and Conover-Iman tests (Figure 2), there were several significant differences in the ARGs abundances on the different days of operation.

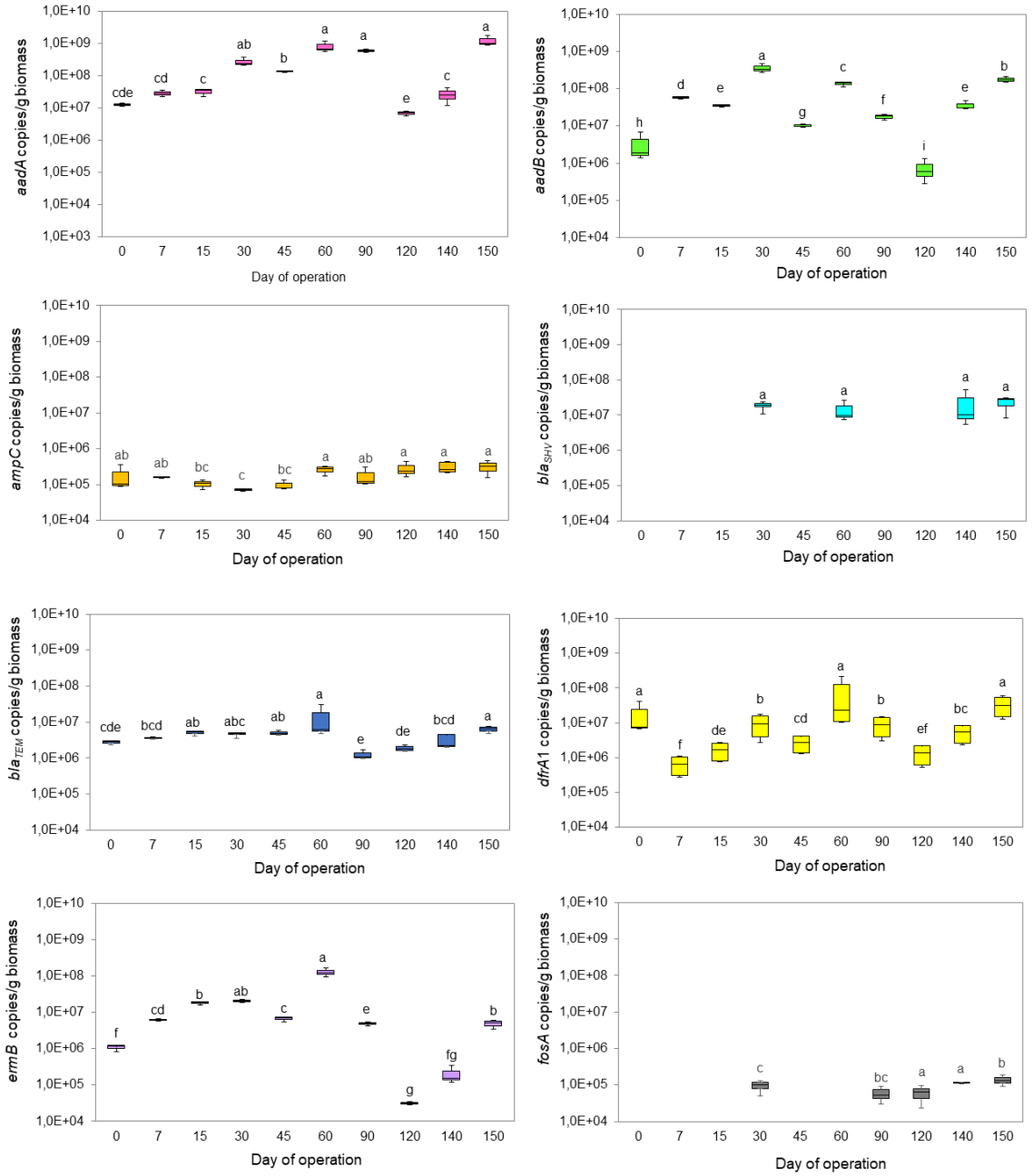


Figure 2. Total abundance of ARGs determine as gene copies per gram of biomass during 150 days of operation by qPCR (n=3). According to the Kruskal-Wallis and Conover-Iman tests ($p < 0.05$) different lowercase letters indicate significant differences among samples for a given ARGs about the day of operation.

The abundance of *ampC* and *dfrA*1 genes was steady between samples during most days of operation. Notably, no statistical differences in their abundance at the beginning of experiment assays (day 0) until the final day of operation (150) were found, suggesting a regular prevalence of the hots bacterial populations under operational conditions here evaluated and the absence of the enrichment of this two genes through the operation. On

the other hand, a significant statistically higher abundance of *aadA*, *aadB*, *bla*$_{SHV}$, *bla*$_{TEM}$, *ermB*, *fosA*, and *qnrS* genes at day 30 compared to those of the initial inoculum was found, suggesting an enrichment of these genes in granular biomass after a short time of operation. Notably, this trend of enrichment was amply observed for *aadA, aadB, bla*$_{SHV}$, *bla*$_{TEM}$, *ermB, fosA,* and *qnrS* at the end of the experiment compared to those values of the inoculum. Therefore, after the time of operation, there was a significant increase of the ARGs abundance in the granular biomass, highlighting the undeniable enrichment of ARB under current AGS operational technology, as it was previously reported (Zhang et al. 2019, Li et al., 2020a, Hu et al., 2024).

In general, the undeniable accumulation of ARGs in sludge-forming biological has been referred as an essential dissemination mechanism to the receiving waterbodies processes in CAS in WWTPs (Tang et al., 2017), activated sludge of more sophisticated systems (Zhang et al., 2013), anoxic-aerobic MBR (Zhu et al., 2018) and anaerobic sequential bioreactor (Gao et al., 2018). However, the theoretically unnecessary purge of the granular biomasses is an essential advantage of the AGS system, reducing the contribution of this process to global antibiotic emergence as it does not require a down-step treatment of the generated sludge compared to the traditional activated sludge. Besides, the long-term endurance and the microbial-self-immobilisation of the granular biomass (Liu et al., 2023) could broadly reduce the dissemination of the ARG to the receiving waterbodies despite their significant enrichment in the biomass). Besides, the broad occurrence of ARGs observed here is in disagreement with the scarce prevalence of human pathogens and potential ARGs host within the granules, mainly *Enterococcus*, *Mycobacterium*, *Dysgonomonas*, members of the family *Clostridiaceae* described (Perez-Bou et al., 2024b). Even though granular biomass exhibited a very robust and resilient microbiome to cope with relevant variations in PhACs of the raw WW inlets (Perez-Bou et al., 2024b), the prevalence of ARGs within granules is still notorious. In this regard, it could be possible that the diversity of the bacteria carrying these ARGs could be found not only in common human pathogens. Also, the high occurrence of antibiotics could enhance a higher number of horizontal gene transfer (HGT) events, increasing the dissemination of these ARGs to bacteria previously sensible. In this way, the contribution of raw WW on the abundance and prevalence of ARGs and ARB have been amply described in several activated sludge samples from WWTPs (Perez-Bou et al., 2024 d) and AGS systems (Wang et al., 2017 b; Li et al., 2020 a,b; Shi et al., 2021).

3.4 ARGs prevalence pattern in relation to bacterial population abundance

The relative abundance of the ARGs, that is the ratio of the different ARGs copies to bacterial 16S rRNA genes, is presented in Figure 3 and Table S3. The highest relative abundances in average values were found for the genes encoding resistance to aminoglycosides (*aadA*, 3.10 % and *aadB*, 0.99%) and to tetracyclines compounds (*tetA*(A), 1.50%) reaffirming their broad persistence in AGS biomass. The remaining genes showed a low relative abundance in average values *bla*TEM (0.49%), *bla*SHV (0.29%), *dfrA*1 (0.20%), *ermB* (0.13 %), and finally *ampC*, *fosA* and *qnrS* (p<0.05%), which might be due to fewer bacterial hosts and mobile genetic elements (MGEs) including these genetic elements for ARGs'proliferation.

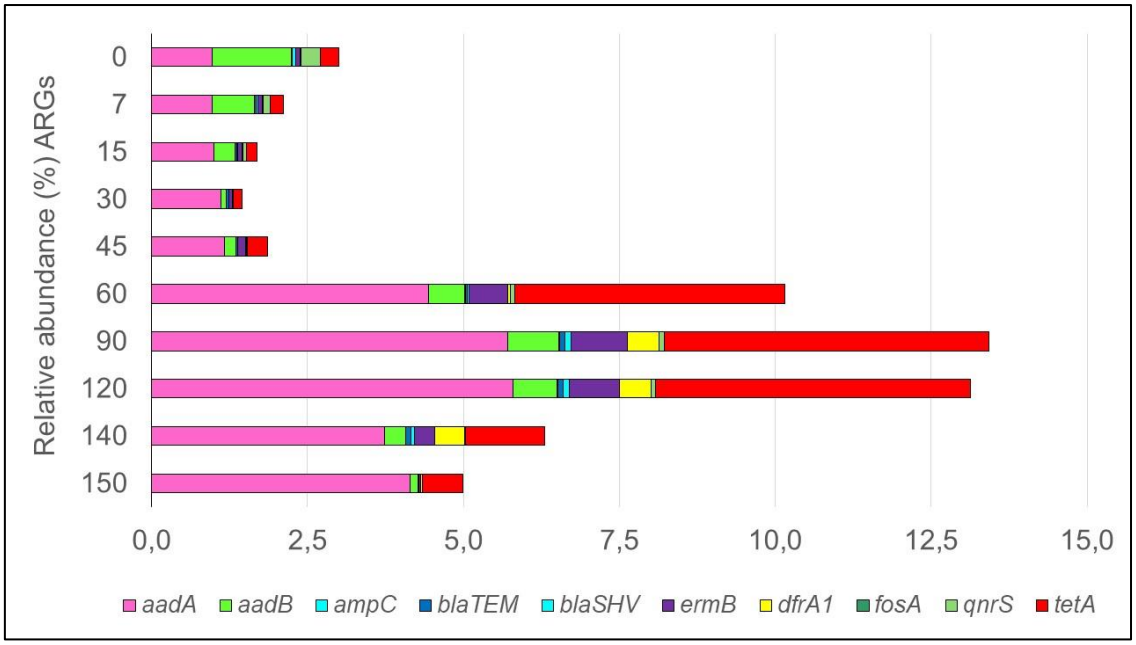


Figure 3. Relative abundance of different ARGs (copies ARG/copies bacterial 16S rRNA) determined by qPCR in samples per day of operation (n=3).

The target ARGs encompass the three major resistance mechanisms-efflux pumps (*tetA* (A)), cellular protection (*dfrA*1, *ermB*, *qnrS*) and antibiotic deactivation (*aadA*, *aadB*, *ampC*, *mecA*, *bla*TEM, *bla*SHV). In general, the resistance mechanism of deactivation of antibiotics is considered to exhibit the highest frequency, which has been confirmed in the AGS laboratory scale (Li et al., 2020a) and in full applications (Tang et al., 2017), as well as in natural environments (Cheng et al., 2020). Besides, efflux pumps are often the first line of defense of bacteria cells against antibiotics, keeping the intracellular levels of antibiotics below inhibitory concentrations (Rahman et al., 2017). After day 60, the

relative abundance of aminoglycoside and tetracycline resistance genes increased. This fact is notably consistent with the highest drug loads in the water inlet previously determined on day 60, which have an obvious effect on the structure of the bacterial populations and the adverse effect on the granule properties, was followed by an evident increase in the relative abundance of ARGs was also evident (fully detailed by Perez-Bou et al., 2024b). The high relative abundance of the *aadA* gene conferring resistance to aminoglycosides has been described as one of the most commonly detected in WWTPs (Nguyen et al., 2021) and also in a full-scale anaerobic-aerobic system treating WW from the industrial production of ribostamycin, spiramycin and paromomycin antibiotics (Tang et al., 2017). Similarly, high levels of the *tetA* genes encoding an efflux pump of tetracyclines, the most common mechanism of tetracycline resistance (Wang et al., 2014) has also been previously suggested (in biological systems (Zhang et al., 2009; Zhang et al., 2013; Xiong et al., 2018) and in anthropogenic sources (Nnadozie and Odume, 2019; Keenum et al., 2022), results in agreement with the found here.

3.5 Correlations of the gene copies of bacterial 16S rRNA and ARGs

The analysis of the Spearman's rank correlations of bacterial 16S rRNA and ARGs total abundance exposed statistically significant correlations among total bacterial abundance and ARGs harbouring bacteria populations (Table 4). Besides, most of the correlations between genes were significantly positive (58.8%). Notably, there was only one significant negative correlation for pair *ermB-fosA*. Also, the high level of significant positive correlations among the different ARGs suggested a strong co-occurrence and potential co-selection events of ARGs coding resistance against different antibiotic classes (Buelow et al., 2020) and stands out the intrinsic relationships of the targeted ARGs within the core of the resistome of the bacterial communities of the granular biomass. In this trend, Li et al. (2020 a,b) highlighted the effects of co-occurrence among ARGs might also influenced the fate of individual ARGs. Then, the dynamic of ARGs patterns depend on the effects of co-selection among ARGs and the evolution of bacterial communities.

The *qnrS* genes were significantly positive correlated with almost all ARGs, except *ampC* and *fosA* genes. It has been described that the *qnrS* gene is a plasmid-mediated resistance within a multi-drug resistance genomic island (Bharathi and Rajamohan, 2022). In this sense, Zhang et al. (2019) reported that increased abundances of *sul*2 and bla_{TEM} were correlated strongly with *tetA* gene, suggesting a co-selection effect attributed to the

physically location on the same mobile genetic element (MEGs, e.g., plasmids, integrons, and transposons) within the genome of the co-hosts. Regarding the importance of MEGs in the ARG transfer, the nucleotidyltransferases genes (*aadA*, *aadB*) exhibited positive correlations with *intI*1, the most widely distributed mobile element in all environments, implying the significance of HGT in their proliferation (Ramirez and Tolmasky, 2010; Tang et al., 2017). Overall, the ARGs encoded by plasmids deserve special attention not only due to their transferability, but also their high level of expression. Hence, further research should monitor the prevalence of MGEs such as integrons and transposons to determine whether ARGs are co-located within the same pathogenic island and predict the expression profile as essential genetic elements favoring the dissemination of ARGs.

Table 4. Spearman's rank correlation coefficients of the abundances of the bacterial 16S rRNA and the different ARGs in samples taken from different day of operation. Absolute values of significant correlations are in bold.

ARGs	*Bacterial 16S rRNA*	*aadA*	*aadB*	*ampC*	*bla$_{TEM}$*	*bla$_{SHV}$*	*dfrA1*	*ermB*	*fosA*	*qnrS*
aadA	0.285									
aadB	***0.698***	***0.585***								
ampC	-0.313	0.221	-0.085							
bla$_{TEM}$	0.260	***0.463***	***0.480***	-0.004						
bla$_{SHV}$	***0.455***	***0.701***	***0.825***	0.202	***0.566***					
dfrA1	0.287	***0.920***	***0.538***	0.202	***0.525***	***0.793***				
ermB	***0.504***	0.267	***0.547***	-0.341	***0.545***	***0.399***	0.318			
fosA	0.066	0.317	0.217	0.148	-0.170	***0.435***	***0.369***	***-0.432***		
qnrS	***0.654***	***0.401***	***0.911***	-0.110	***0.560***	***0.777***	***0.418***	***0.719***	0.019	
tetA(A)	-0.023	0.302	0.096	-0.131	-0.081	0.113	0.334	***0.501***	-0.212	0.150

3.6 Correlation of abundance ARGs under the antibiotic occurrence

To determine the influence of antibiotics on the proliferation of ARGs a Spearman's rank correlation analysis was also carried out (Table 5). No significant correlations were found between the abundance of bacterial populations and the occurrence of antibiotics in the raw water inlet. A plethora of strong positives correlation values were observed for some antibiotics and the ARGs, including those that confer antibiotic resistance to them, such as CIP-*qnrS*, ERY-*ermB*, GEN-*aadA* and *aadB*, reaffirming the potent selecting effect of the presence of the antibiotics on ARGs prevalence, even for non-corresponding antibiotics, which have been referred in AGS reactors (Zhang et al., 2019 b; Hu et al., 2024). Liu et al. (2019) theorised that a high presence of plasmid-borne ARGs, such as

aminoglycoside, sulfonamide, and tetracycline resistance genes, could be associated with the presence of target antibiotics. Accordingly, Zhang et al. (2019) reported that tetracycline exposure served as a co-selection pressure for ARGs corresponding to other types of antibiotics in a AGS reactor.

Table 5. Spearman rank order correlations of abundance of Bacterial 16S rRNA and ARGs under antibiotic occurrence in influents (INF) samples.

	Bacterial 16S rRNA	*aadA*	*aadB*	*ampC*	*bla*$_{TEM}$	*bla*$_{SHV}$	*dfrA*1	*ermB*	*fosA*	*qnrS*	*tetA*(A)
AMP_INF	0,043	-0,351	**-0,409**	**-0,427**	0,090	-0,091	0,133	-0,259	0,101	-0,217	-0,113
AZN_INF	0,319	0,143	**0,557**	-0,105	0,237	**0,620**	0,210	0,326	0,032	**0,648**	0,341
CIP_INF	0,090	0,304	**0,538**	0,150	**0,428**	**0,606**	0,234	**0,447**	-0,180	**0,649**	**0,406**
CLM_INF	-0,384	-0,163	0,054	0,014	0,036	0,325	-0,266	-0,008	**0,441**	0,289	-0,102
DOX_INF	0,083	**-0,418**	-0,399	0,236	-0,297	-0,382	-0,015	-0,312	-0,173	**-0,483**	-0,299
ERY_INF	0,218	**0,521**	**0,769**	-0,269	0,344	**0,589**	0,050	**0,561**	0,147	**0,772**	0,378
GEN_INF	0,184	**0,426**	**0,780**	0,184	**0,444**	**0,703**	0,163	**0,640**	-0,169	**0,804**	0,381
SMX_INF	-0,100	0,267	**0,634**	0,266	0,128	**0,493**	-0,205	0,385	0,131	**0,623**	0,121
TMP_INF	0,011	0,103	0,072	-0,169	**0,543**	-0,301	-0,198	0,317	**-0,694**	0,134	-0,277

Similarly, SMX and TC occurrence promoted the HGT phenomenon and increased the resistant proportion of the bacterial community by 0.5-1.4 orders of magnitude on the bacterial community in an MBR system treating synthetic WW (Zhu et al., 2018). Similarly, Chen et al. (2019) reported the grateful influence of TC on increase *intI*1 and tetracycline resistance genes in the sludge by 4.25 times and 4.7-186.9 times, respectively. Notably, the abundance of no-corresponding ARGs to different types of antibiotics in AGS can significantly rise under the presence of antibiotics, as well has been demonstrated in SDZ and the abundances of sulfanilamide resistance genes (raised on 2-3 times) in biomass (Wan et al., 2018) and tetracycline and the abundances of *tetA*, *sul*2 and *bla*$_{TEM}$ (Zhang et al., 2019 b) and ENR in the abundances of tetracycline and aminoglycoside resistance genes in AGS biomass (Li et al., 2020b). However, Shi et al. (2022) observed an absence of accumulation of *qnrS* gene when an AGS reactor was operated under the presence of different fluoroquinolones (CIP, OFL, and NOR) at various doses (3300 and 900 μg L^{-1}).

On the other hand, there was no relation between high concentrations of SMX/TMP with abundance of *dfrA*1 gene. In the same trend, no significant relationship was found between the occurrence of AMP class with the prevalence of *bla*$_{TEM}$ and *bla*$_{SHV}$.

Nevertheless, the high load of CIP, SMX, ERY and GEN modulated the prevalence of the no-target ARGs in this study, since an amply co-selection phenomenon of non-corresponding ARGs could be derived. A solid number of negative correlations were also found between antibiotics (AMP, CLM, DOX, ERY, and TMP) and ARGs by exerting a cellular stress and inhibition effect in the bacterial population carrying these genes, reinforcing the pivotal role played by distinct antibiotics in the resistome of bacterial communities and the co-selection of ARGs (Hu et al., 2024).

In agreement with the strong positive Spearman's rank correlation coefficients of qnrS with bacterial 16S rRNA, *aadA*, *aadB*, *bla*$_{TEM}$, *bla*$_{SHV}$, *dfrA*1 and *ermB* genes (Table 4), the correlation with the *qnrS* gene and antibiotic compounds occurrence showed the same trend (Table 5). This agrees well with the dissemination of plasmid-mediated resistance carrying the *qnrS* gene, in which the high presence of the target antibiotic provides a favorable background for the selection and proliferation of other additional resistance mechanisms. Given the accelerating emergence of antibiotic resistance as a critical public issue and environmental health concern, limiting the impact of antimicrobial resistance is essential today (Aslam et al., 2021; Abbas et al., 2024). As a strategy to achieve this goal, is to reduce exposure and increase the removal rates of antimicrobial compounds, the optimisation of operating conditions has been proposed as a key factor (Kang et al., 2018b; Wang et al., 2022). Therefore, understanding the dynamics of ARGs in biological systems may help to develop strategies to mitigate their proliferation and spread, especially when real WW is used.

In this respect, this study highlighted the feasibility of the *in situ* hospital WW treatment based on AGS systems, helping to meet the current global challenge of WW treatment. Also, the pivotal role played by antibiotics in the shaping of the resistome and the co-selection of ARGs has been highlighted. Nevertheless, despite the advantages of AGS system, it is essential to limit the accumulation of ARGs for the implementation of AGS technology for full-scale WW treatment strategies, as it has been described as a critical concern for the performance of biological treatment(Li et al., 2020a). Furthermore, considering the fact that the spread of ARGs is still undeniable under current AGS operating conditions (Zhang et al., 2019 b; Li et al., 2020a; Hu et al., 2024) and even more so under the antibiotic occurrence (Amorim et al., 2014), the contribution of AGS to their spread needs to be addressed in further studies. Finally, recent research has demonstrated that many non-antibiotic chemicals, such as microplastics, metallic

nanoparticles and non-antibiotic drugs, can accelerate the dissemination of ARGs (Shi et al., 2022). Therefore, the role and mechanisms of non-antibiotic chemicals and related environmental factors must be fully understood to design and implement politics aimed to reduce the contribution of WW and particularly hospital WW, in the antibiotic resistance matter.

4. CONCLUSIONS

This study addressed the viability of the AGS technology to *in situ* treat real hospital WW with high and variable concentrations of antibiotics, showing adequate performance for removing antibiotics of different classes found in the hospital's raw wastewater. Notably, broad removal rates, nearest 90% of elimination, were achieved punctually for AMP, AZN, CLM, DOX, ERY, GEN, SMX, and TMP. However, CIP, the antibiotic with the highest concentration, showed a recalcitrant removal pattern as only a moderate elimination rate was achieved due to a desorption process. On the other hand, the successful application of the new qPCR protocols highlights the prevalence of *aadA*, *aadB, ampC, bla*$_{\text{TEM}}$, *dfrA1*, and *ermB* in hospital influents. Similarly, the most abundant genes in the granular biomass were those ARGs conferring resistance to aminoglycosides (*aadA*, *aadB*), tetracyclines (*tetA*(A)), macrolides (*ermB*), pyrimidines (*dfrA*1) and quinolones (*qnrS*). Besides, the high co-occurrence of antibiotics and ARGs suggests that the high exposure to antibiotics resulted in the accumulation of *aadA*, *aadB*, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *ermB*, *fosA* and *qnrS* resistance genes, reaffirming the outstanding contribution of hospital WW to the spread and proliferation of the antimicrobial resistance phenomenon. Hence, this research contributed to expanding the applicability of AGS system for the *in situ* treatment of real hospital WW as a promising technology with broad adaptability and excellent performance to reduce the contribution of these effluents to the global antibiotic resistance emergence before disposal to the municipal sewage system.

SUPPLEMENTARY MATERIAL

Dynamic of antibiotic resistance genes under the antibiotic occurrence in aerobic granular sludge system treating real hospital wastewater

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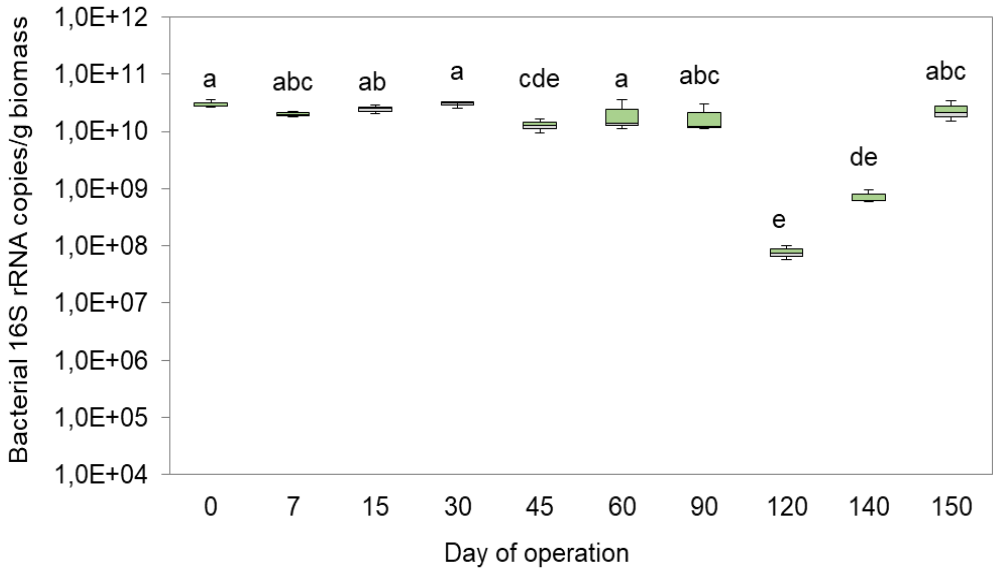

Figure S1. Box–and-Whiskers plots of the abundances of the bacterial 16S rRNA genes determined by quantitative PCR in granular biomass samples (n = 3). Different lowercase letters indicate significant differences among samples, according to the Kruskal-Wallis and Conover-Iman tests ($p < 0.05$).

Table S3. Relative abundances of different ARGs (copies ARG/copies bacterial 16S rRNA) determined by quantitative PCR in samples taken from different days of operation (n = 3). According to the Kruskal-Wallis and Conover-Iman tests ($p < 0.05$). Different lowercase letters indicate significant differences among the gene copies of the different ARGs for a given sample.

Day	*aadA*	*aadB*	*ampC*	*bla*$_{TEM}$	*bla*$_{SHV}$	*dfrA1*	*ermB*	*fosA*	*qnrS*	*tetA*(A)
0	0.04 ^{f}	0.01 ^{f}	n.d.	0.01 ^{e}	n.d.	0.06 ^{de}	n.d.	n.d.	n.d.	0.15 ^{d}
7	0.14 ^{e}	0.29 ^{d}	n.d.	0.02 ^{cd}	n.d.	n.d.	0.03 ^{e}	n.d.	0.02 ^{d}	0.03 ^{e}
15	0.13 ^{e}	0.14 ^{e}	n.d.	0.02 ^{cd}	n.d.	n.d.	0.08 ^{b}	n.d.	0.01 ^{e}	0.02 ^{e}
30	0.97 ^{d}	1.27 ^{b}	n.d.	0.02 ^{d}	0.06 ^{b}	0.02 ^{fg}	0.07 ^{b}	n.d.	0.31 ^{a}	0.30 ^{c}
45	1.12 ^{d}	0.08 ^{ef}	n.d.	0.04 ^{b}	n.d.	0.01 ^{g}	0.06 ^{bc}	n.d.	n.d.	0.14 ^{d}
60	5.70 ^{bc}	0.83 ^{bc}	n.d.	0.10 ^{b}	0.10 ^{b}	0.51 ^{bc}	0.90 ^{a}	n.d.	0.09 ^{b}	5.20 ^{b}
90	4.15 ^{bc}	0.13 ^{e}	n.d.	0.01 ^{e}	n.d.	0.03 ^{ef}	0.03 ^{de}	n.d.	n.d.	0.65 ^{c}
120	9.26 ^{a}	1.07 ^{bc}	0.35 ^{a}	2.62 ^{a}	n.d.	0.92 ^{a}	0.04 ^{cd}	0.07^{a}	n.d.	6.16 ^{a}
140	3.59 ^{c}	5.26 ^{a}	0.04 ^{a}	2.04 ^{a}	2.68 ^{a}	0.38 ^{ab}	0.03 ^{ef}	0.02 ^{a}	0.06 ^{bc}	2.35 ^{b}
150	5.91 ^{ab}	0.80 ^{c}	n.d.	0.03 ^{bc}	0.09 ^{b}	0.09 ^{cd}	0.02 ^{f}	n.d.	0.05 ^{cd}	n.d.

CHAPTER-IV

Abstract

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Cold environments are the most widespread extreme habitats in the world. However, the role of wastewater treatment plants (WWTPs) in the cryosphere as hotspots in antibiotic resistance dissemination has not been well established. Hence, a snapshot of the resistomes of WWTPs in cold environments, below 5 °C, was provided to elucidate their role in disseminating antibiotic resistance genes (ARGs) to the receiving waterbodies. The resistomes of two natural environments from the cold biosphere were also determined. Quantitative PCR analysis of the *aadA*, *aadB*, *ampC*, bla_{SHV}, bla_{TEM}, *dfrA*1, *ermB*, *fosA*, *mecA*, *qnrS*, and *tetA*(A) genes indicated strong prevalences of these genetic determinants in the selected environments, except for the *mecA* gene, which was not found in any of the samples. Notably, high abundances of the *aadA*, *ermB*, and *tetA*(A) genes were found in the influents and activated sludge, highlighting that WWTPs of the cryosphere are critical hotspots for disseminating ARGs, potentially worsening the resistance of bacteria to some of the most commonly prescribed antibiotics. Besides, the samples from non-disturbed cold environments had large quantities of ARGs, although their ARG profiles were highly dissimilar. Hence, the high prevalences of ARGs lend support to the fact that antibiotic resistance is a common issue worldwide, including environmentally fragile cold ecosystems.

Keywords: Cold environments, cryosphere, antibiotic resistance, ARGs, ARB, qPCR, multi-drug resistance, WWTPs, activated sludge, influent sewage

CHAPTER IV: Deciphering the role of WWTPs in cold environments as hotspots for the dissemination of antibiotic resistance genes

1. INTRODUCTION

Approximately 80% of the Earth's biosphere is considered a cold habitat, that is, with average annual temperatures below 5 °C (De Maayer et al., 2014). Hence, cold-temperature environments are the most widespread extreme habitats, including most of Canada, the northern USA, northern Europe, Russia, and northern China polar areas, high-mountain lakes, and most oceans (De Maere et al., 2013). Psychrophile microorganisms have adapted to cope with temperature stress by diverse mechanisms to support cellular membrane homeostasis and biochemical catalysis (Zhou et al., 2018) by producing cryoprotective exopolysaccharides, increasing membrane fluidity, and biosynthesising enzymes that retain the functionality in these cold temperatures (Maza-Márquez et al., 2022). These microorganisms also must deal with low nutrient levels, salinity stress, desiccation, and different light conditions among seasons (La Para et al., 2015; Caucci et al., 2016; Gonzalez-Martinez et al., 2018). Hence, cold temperatures play a pivotal role in the bacterial communities of the cryosphere, resulting in a wide selection and adaptation of the resident microorganisms within these habitats and, therefore, in their functional diversification (Boetius et al., 2015). Psychrophilic bacteria represent the most abundant, diverse, and widely distributed extremophiles on Earth, driving critical global biogeochemical cycles. Therefore, microbial communities in low-temperature environments are the single largest biome on Earth (Shen et al., 2021). In addition, these extreme habitats tend to be highly fragile and vulnerable to human activities, making it necessary to protect them from environmental contamination (Sayed et al., 2020).

The broad misuse or overuse of antibiotics for humans and livestock has led to the development and proliferation of antibiotic-resistant bacteria (ARB), whose abundances have increased up to 15 times in the last years (García et al., 2020). The wide distribution of ARB has been related to approximately 25,000 and 23,000 annual deaths in Europe and the USA, respectively (Lorenzo et al., 2018). It has been estimated that by 2050, the proliferation of ARB will result in 10 million deaths worldwide (Tagliabue and Rappuoli, 2018). Furthermore, the dissemination of antibiotic resistance genes (ARGs) has resulted

in the ubiquitous detection and accelerated proliferation of ARB in several environments (Johnson et al., 2019). Antibiotic accumulation can also negatively impact water pH, conductivity, total hardness, and nitrogen homeostasis (Zhu et al., 2022b), with negative consequences for the biodiversity and functional stability of the microbial communities (Ben et al., 2019). Hence, antibiotic resistance represents a serious threat to ecosystems and human health (WHO, 2014; WHO, 2020), making it mandatory to minimise ARG enrichment in the bacterial communities of diverse ecosystems to ameliorate the emergence of antibiotic resistance.

Wastewater treatment plants (WWTPs) are considered a source of antibiotic pollution, ARB dissemination hotspots, and ARGs reservoirs (Guo et al., 2017; Karkman et al., 2018). Mainly, the large nutrient amounts and high bacterial abundances in WWTPs widely facilitate the proliferation and development of ARGs by horizontal gene transfer (HGT) from multi-drug-resistant to antibiotic-sensitive bacteria (Shao et al., 2018). Several studies have therefore analysed the ARGs' prevalence in influents, activated sludge, and effluents of WWTPs worldwide (Rodriguez-Mozaz et al., 2015; Zhu et al., 2018; Ju et al., 2019; Chiemchaisri et al., 2022). In this regard, the high prevalence and persistence of ARGs in WWTP effluents after biological treatment have been amply described (Marti et al., 2013 a,b; Tong et al., 2019; Yu et al., 2020; Yin et al., 2021). Hence, the discharged effluents after wastewater (WW) treatment are a critical source of ARGs as these genetic determinants can be easily transferred to the receiving waterbodies and further to distant surface waters, sediment, and soils, increasing antibiotic resistance (Cahill et al., 2019; Delgado-Blas et al., 2021).

Traditionally, the analysis of the ecophysiology of bacterial communities has relied on cultivation-based methods, allowing the isolation of several bacteria from various extreme habitats (Chanal et al., 2006; Pettit, 2011). Nevertheless, in the last years, the advent of different cultivation-independent surveys has allowed the identification of the microbial diversity previously not represented in the microorganisms available in pure cultures (Tighe et al., 2017; Marti et al., 2013 a,b). In this sense, the technological development of culture-independent methods, mainly real-time PCR (quantitative PCR, qPCR) analysis, has been broadly employed to obtain an understanding of the ecology of bacterial communities in extremely low-temperature environments (Zhu et al., 2015; Zhang et al., 2019c). For that matter, numerous studies have addressed the microbial diversity and ecology of psychrophilic bacterial communities in several cold

environments (Boetius et al., 2015; Cavicchioli, 2015; Fang et al., 2018; Perini et al., 2019), according to their extension in the Earth's cold biosphere and their relevance to global biogeochemical cycles. Remarkably, several studies have addressed the bacterial communities of WWTPs in zones with average low temperatures (northern Europe, Asia, and North America) due to the necessity of treating the urban and industrial WW generated in these cold regions (Fang et al., 2018; Xu et al., 2018; Luo et al., 2020). Notably, it has been described that the microbial communities of activated sludge in WWTPs in cold environments are amply affected by the low temperature, which could lead to a deterioration of WW treatment performance due to low microbial activity, substrate utilisation, and removal rate of ammonia nitrogen (Fang et al., 2018; Luo et al., 2020).

In addition, the low water temperatures of these WWTPs decrease activated sludge floc and sludge settleability performance due to increased abundances of filamentous microorganisms, leading to the deterioration of the performance of processes (Luo et al., 2020). However, different studies have demonstrated that the psychrophilic bacterial communities achieved adequate levels of adaptation to low-temperature operation conditions under different configurations of WWTPs, allowing successful performances in terms of organic matter and nutrient removal efficiencies despite the inhibited metabolism of mesophilic microorganisms (Fang et al., 2018; Rodriguez-Sanchez et al., 2020; Maza-Márquez et al., 2022). In this regard, an overabundance of bacteria that can catabolise refractory organic compounds and more abundant phosphorus removal bacteria were found in psychrophilic activated sludge (Fang et al., 2018). In addition, the abundances of heterotrophic bacteria and functional populations (ammonium oxidising bacteria, nitrite oxidising bacteria, and phosphate accumulating bacteria) do not have significant differences in the community structures at the phylum and class levels compared with those of other sewage treatment processes in middle-warm climates, although they differed in the dominant genera (Luo et al., 2020; Xu et al., 2020).

Finally, it has been recently revealed that a high functional diversity of mechanisms against cold shock by metatranscriptomics analysis are cold shock proteins type A (CspA), trigger factors, antitermination proteins type *NusA*, polyribonucleotide nucleotidyltransferases, cytochrome C oxidases, ATP synthases, transposases, ABC transporters, permeases, catalases and superoxide dismutases, UV radiation resistance proteins (UvrA), and chaperones (Maza-Márquez et al., 2022).

On the other hand, the occurrence and abundance of ARGs of the psychrophilic bacterial communities of activated sludge of WWTPs in cold environments remain widely unknown, calling for studies on the presence of ARGs of WWTPs operating at low temperatures and for the evaluation of their capacity to disseminate ARGs into their receiving waterbodies and, consequently, into pristine environments. Hence, determining the occurrences and abundances of ARGs during WW treatment in cold areas is of great importance to prevent the dissemination of ARB and ARGs to the receiving aquatic ecosystems.

This study provides a snapshot of the occurrence of ARGs of WWTPs in cold environments and elucidates their potential role as hotspots in the dissemination of ARGs compared to the prevalence of ARGs in pristine environments of the cold biosphere. For that purpose, the abundances of several ARGs in different WWTPs from Finland were determined by using recently developed qPCR methodologies that allow a total coverture of the biodiversity of these ARGs. The most prescribed antibiotics in Finland are beta-lactams (70%), tetracyclines (12%), macrolides, lincosamides, and streptogramins (7%), sulfonamides and trimethoprim (TMP) (5%), and quinolones (5%) (Pyörälä et al., 2022). The selection of the *aadA*, *aadB*, *ampC*, *bla*$_{SHV}$, *bla*$_{TEM}$, *dfrA*1, *ermB*, *fosA*, *mecA*, *qnrS*, and *tetA*(A) genes was made according to their clinical importance and incremented resistance to the corresponding antibiotics in WWTPs, covering the commonly worldwide and locally used antibiotics according to their chemical structures (aminoglycoside, beta-lactams, macrolide, quinolone, sulphonamide/TMP, and tetracycline) (Nguyen et al., 2019; Wang et al., 2020; Pyörälä et al., 2022). In addition, such results would shed light on the characterisation of the abundance of important ARGs of WWTPs in cold environments and supply theoretical explanations for the role of psychrophilic activated sludge of WWTPs as essential sources of ARGs and hotspots of the dissemination of ARB to fragile receiving ecosystems.

2. MATERIAL AND METHODS

2.1 Biomass source

For this study, influent and activated sludge samples were collected from five municipal WWTPs located in different geographical locations in Finland within the Polar Arctic Circle: Ruka, Sirkka, Rovaniemi, Ylitornio, and Kemijärvi WWTPs. Ruka WWTP is a full-scale MBR with an operating temperature of 5.0 °C located in the ski station of

Rukatunturi (Kuusamo, Pohjois-Pohjanmaa Region, mean annual temperatures (MAT) of 1.0 °C (https://weath erspark.com/)). Sirkka WWTP is a conventional activated sludge (CAS) system with a process temperature of 5 °C to treat the WW generated in the Levi Ski Station (Kittilä, Lapin Region, MAT of 4.5 °C). Rovaniemi WWTP is a CAS system operating at 4.0 °C located on Ala-Korkalo to treat the sewage generated in Lapin Capital (MAT of 1.1 °C and 63,128 inhabitants). Ylitornio WWTP is a small CAS system with an operating temperature of 7 °C to treat the sewage generated by the 4850 inhabitants of this Lapin municipality (MAT 0.1 °C). Finally, Kemijärvi WWTP is based on activated sludge technology with simultaneous phosphorus precipitation and operating temperatures of 4.0 °C in the Kemijärvi city (Lapin Region) with a MAT of 0.7 °C and 6995 inhabitants. Full details of the WWTPs are described in Gonzalez-Martinez et al. (2018) and Rodriguez-Sanchez et al. (2020). In addition, non-disturbed soil samples from the Grábrók Volcano near Bifröst (Iceland) and sediment samples from the Yeguas Lake (Sierra Nevada, Granada, Spain) were taken as natural cold-environment controls (with average annual temperatures of 1.6 °C and 3.9 °C, respectively).

2.2 DNA extraction

Total DNA was simultaneously extracted from 0.5 g of each biomass sample using the FastDNA SPIN Kit for soil and the FastPrep 24-Instrument (MP Biomedicals, Germany) as described elsewhere (Correa-Galeote et al., 2021). The DNA obtained was eluted in 200 µL of DNase/pyrogen-free water and stored at - 20 °C until further use. The quality of the extracted DNA was checked by electrophoresis on 1% agarose and quantified by spectrophotometry at 260 nm using a Nanodrop ND1000 spectrophotometer (Thermo Fisher Scientifc, USA).

2.3 Quantitative polymerase chain reaction

Total bacteria and ARGs were determined via qPCR on a QuantStudio-3 Real-Time PCR system (Applied Biosystems, USA). The bacterial 16S rRNA genes were amplified to estimate the total bacterial population abundances using the primers 341F and 534R as elsewhere described (Correa-Galeote et al., 2021). Quantification of the ARGs from environmental samples was also made, including *aadA* and *aadB* (as proxies to determine aminoglycosides resistance); *ampC*, bla_{TEM}, bla_{SHV}, and *mecA* (as agents to evaluate beta-lactams resistance); *dfrA*1 (as a representative to analyse TMP resistance); *ermB* (as a proxy to assess macrolide resistance); *fosA* (as a target to quantify fosfomycin resistance);

qnrS (as an agent to measure quinolone resistance); and *tetA*(A) genes (as a proxy to establish tetracycline resistance). The qPCR conditions, methodologies, and thermal profiles can be found in Perez-Bou et al. (2024b). These authors recently developed a set of primers that allow higher covertures of the different ARG sequences than those of the currently available molecular tools, more accurately reflecting the total abundances of the ARGs in various environmental samples. The reactions (two independent experiments, three replicates per DNA sample) were performed in 25 µL of reaction mixture as described elsewhere (Correa-Galeote et al., 2021). As standards for quantifying the respective target genes, dilution series of cloned linear fragments, developed previously, were used (Perez-Bou et al., 2024 d; Correa-Galeote et al., 2021). The qPCR calibration curves were constructed with the plasmid standard using serial tenfold dilutions (10^2-10^8), with correlation coefficients (R^2)>0.99 in all qPCR assays and PCR efficiencies ranging between 80 and 105%.

2.4 Statistical analysis

The statistical differences were analysed using the Kruskal-Wallis and Conover-Iman combined tests and the Mann-Whitney test ($p < 0.05$ significance level) in XLSTAT v. 2020 (Addinsoft, USA). Additionally, correlation analysis of the abundance of the different ARGs was made using Spearman's rank correlation coefficients, employing the XLSTAT software.

3. RESULTS AND DISCUSSION

3.1 Total bacterial populations

The gene copies of the 16S rRNA gene were determined by qPCR to assess the total abundance of bacteria, as summarised in Figure 1. Although there were significant differences in the concentrations of bacterial 16S rRNA gene among the influents and activated sludges of the different WWTPs, the total abundances of bacteria are in agreement with those previously described for full-scale WWTPs (Gonzalez-Martinez et al., 2018; Rodriguez-Sanchez et al., 2020) and lab-scale experiments (Gonzalez-Martinez et al., 2017; Muñoz-Palazon et al., 2022 a,b) operating at low temperature. Similarly, the values of total bacterial abundances were in the same range as those of other real WWTPs at non-cold temperatures (Zhou et al., 2018; Abzazou et al., 2018; Wei et al., 2018; Niestępski et al., 2020). Particularly, the reduced values of the bacterial abundance in the influent from the Sirkka WWTP must be mentioned, a fact that could be related to the

specific conditions of this WWTP that treat the WW generated in a Ski Station, although standard values of organic contaminants have been previously described for this WWTP (Gonzalez-Martinez et al., 2018).

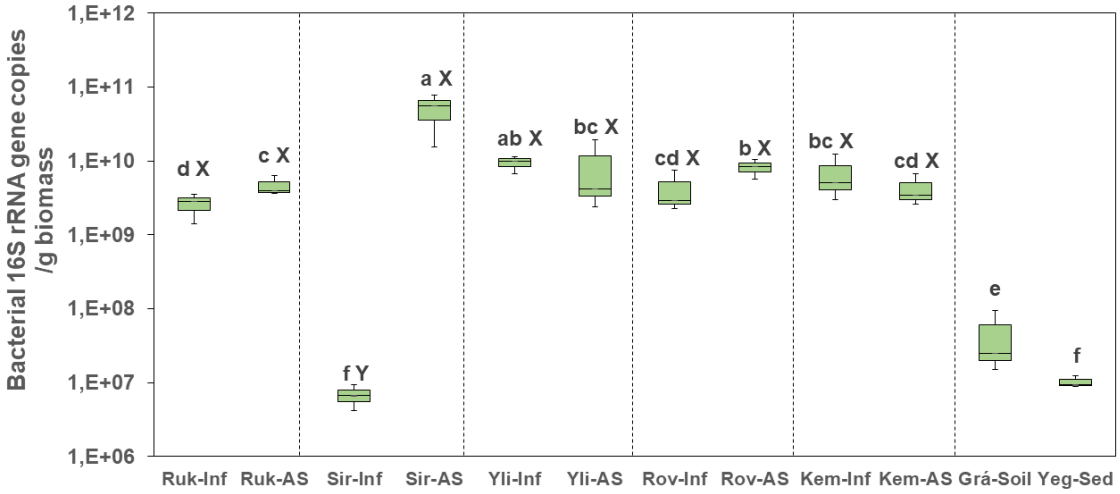

Figure 1. Box–and Whiskers plots of the abundances of the bacterial 16S rRNA genes determined by quantitative PCR in samples taken from cold environments (n = 6). Different lowercase letters indicate significant differences among samples, according to the Kruskal-Wallis and Conover-Iman tests ($p < 0.05$). Different capital letters indicate significant differences between influent and activated sludge samples of a given WWTP, according to the Mann-Whitney test ($p < 0.05$). Ruk-Inf, influent taken from Rukka WWTP, Ruk-AS, activated sludge of Rukka WWTP, Sir-Inf, influent taken from Sirkka WWTP, Sir-AS, activated sludge of Sirkka WWTP, Yli-Inf, influent taken from Ylitornio WWTP, Yli-AS, activated sludge of Ylitornio WWTP, Rov-Inf, influent taken from Rovaniemi WWTP, Rov-AS, activated sludge of Rovaniemi WWTP, Kem-Inf, influent taken from Kemijarvi WWTP, Kem-AS, influent taken from Kemijarvi WWTP, Grá-Soil, Grábrók Volcano soil, Yeg-Sed, Yeguas Lake sediment.

Some authors have highlighted that low temperatures significantly decreased WW treatment performance and impeded sludge settleability, mainly inhibiting microbial activity and metabolism and increasing the presence of undesirable filamentous bacteria (Winkler et al., 2012; Gnida et al., 2016; Zhou et al., 2018; Yuan et al., 2022). However, others have shown successful operations of WWTPs in cold regions, achieving adequate levels of biomass and a good pollutant removal efficiency (Xu et al., 2018; Li et al., 2020c). In agreement with the latter, the results of the present study highlight the successful establishment of appropriate bacterial communities in terms of biomass, allowing a proper WW treatment performance despite the negative impact of cold

temperatures on bacterial growth and metabolism. On the other hand, the total bacterial abundances in the natural environments (Grábrók Volcano soil and Yeguas Lake sediment) reported here were lower than those described in other samples from non-anthropogenically disturbed cold environments (Petro et al., 2019); however, the values found in this study were higher than those determined in other samples' natural cold-ecosystems (Gokul et al., 2019). Henceforth, the bacterial communities of the cold environments present a broad range of abundances, indicating that the specific properties of these ecosystems highly modulate the quantities of the bacterial communities.

3.2 Occurrence of genetic determinants conferring resistance to antibiotics

Among the samples from the different cold environments analysed, all tested genetic determinants responsible for the resistance to different antibiotic compounds were detected by the corresponding qPCR analysis, except for the *mecA* gene (genetic determinant encoding a penicillin-binding protein with reduced affinity for beta-lactams and with a high prevalence in hospital environments (Shoaib et al., 2020), which was not detected in any of the samples (Figure 2).

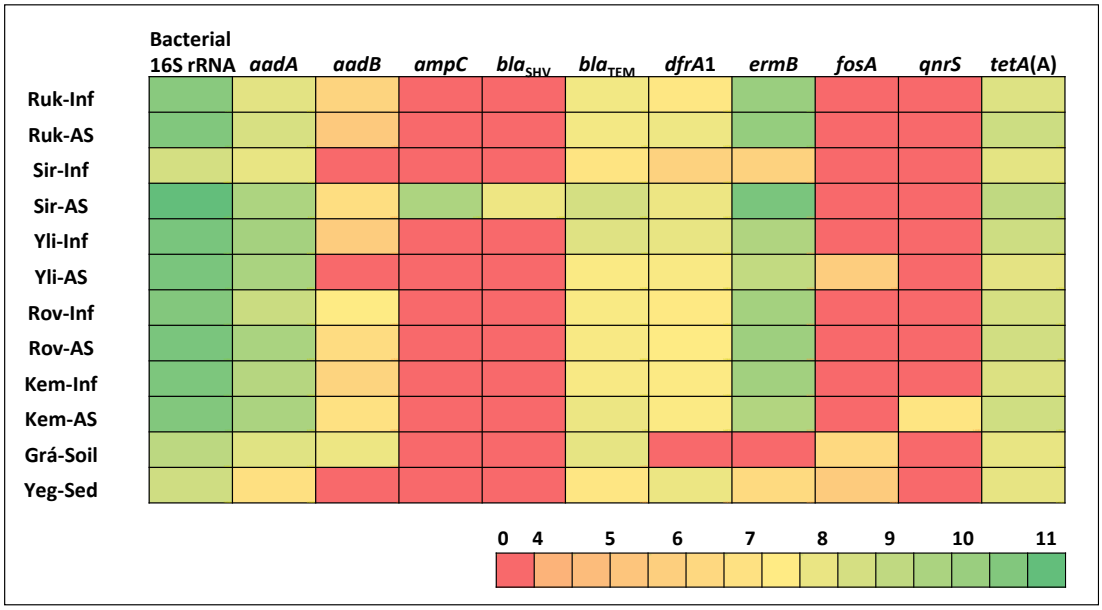

Figure 2. Heatmap of the gene copies (in logarithmic scale) of the targeted marker genes (bacterial 16S rRNA and the different ARGs (*aadA*, *aadB*, *ampC*, *bla*$_{SHV}$, *bla*$_{TEM}$, *dfrA*1, *ermB*, *fosA*, *mecA*, *qnrS*, and *tetA*(A) genes) per gram of biomass, quantified by quantitative PCR in samples taken from cold environments

The *ampC* and *bla*$_{SHV}$ genes were only found in the activated sludge of the Sirkka WWTP; similarly, the *qnrS* gene was only detected in the activated sludge of Kemijarvi WWTP.

In addition, the *fosA* gene was only found in the Ylitornio WWTP and both natural controls (Grábrók Volcano soil and Yeguas Lake sediment). The *aadB* gene was not found in the influent samples from Sirkka and Ylitornio WWTPs and in Yeguas Lake, and the *dfrA*1 gene was found in all samples except in the lava soil taken from Grábrók Volcano. The remaining genes (*aadA*, *bla*$_{TEM}$, and *tetA*(A)) were found in all samples analysed. The occurrence stability indices of the gene copy values per gram of biomass were also calculated (Lucena-Padrós et al., 2019). Accordingly, the beta-lactam resistant *ampC* and *bla*$_{SHV}$, and *qnrS* genes, encoding a pentapeptide repeat protein that inhibits the action of fluoroquinolone on bacterial DNA gyrase and topoisomerase IV (Robicsek et al., 2006), were considered accidental genetic determinants. The presence of the *fosA* gene, encoding an inactivating fosfomycin enzyme (Güneri et al., 2022) and the genes *aadA* and *aadB*, which produce aminoglycoside-modifying enzymes (Aliakbarzade et al., 2014), was accessory. The *bla*$_{TEM}$, encoding extended-spectrum beta-lactamases (ESBL) (Algammal et al., 2020), *dfrA*1, coding for an insensitive dihydrofolate reductase conferring resistance to TMP (Lombardo et al., 2016), *ermB*, encoding ribosomal RNA methylases that modify the target sites of macrolides (Qin et al., 2014), and *tetA*(A), producing a tetracycline antiporter (Bertram et al., 2022), were euconstants in the ARG profiles of the different samples.

3.3 Total abundances of antibiotic resistance genes

The total abundances of the different ARGs among all cold environment samples oscillated from below the limit of quantification (<10^3 copies/g of biomass) to 10^9 copies/g of biomass (Figure 3). Hence, ARG abundances were in the same range as those previously described for different environments such as WWTPs (Wen et al., 2016; Quintela-Baluja et al., 2019), soils (Zhang et al., 2017b; Liu et al., 2022), and sediments (Li et al., 2018; Wang et al., 2021). This study supports evidence that the global dissemination of ARGs and ARB is a growing phenomenon, even in environments from cold and pristine areas with small urban nuclei, as previously depicted (Miller et al., 2014; Sun et al., 2021). In this regard, the expression of the *aadA* and *aadB* genes confers resistance to aminoglycosides antibiotics in microorganisms carrying extensive antibiotic resistance within WW (Weist et al., 2016; Selvaraj et al., 2022: Ambrose et al., 2023). The *ampC* gene and the *bla*$_{SHV}$ and *bla*$_{TEM}$ gene enzymes encoding AmpC beta-lactamases and extended-spectrum beta-lactamases, respectively, inactivate most broad-spectrum beta-lactam antimicrobials which are highly prevalent in WWTP influents as

domestic, industrial, and clinical sewage (Schages et al., 2020). In addition, the penicillin-binding protein (PBP2a) encoded by the *mecA* gene confers resistance to mainly all beta-lactam compounds except for ceftaroline and other 5th-generation cephalosporins, a resistance mechanism yet circumscribed basically to healthcare environments (Liao et al., 2018). The expression of the *dfrA*1 gene inhibits the combination of TMP/SMX; antibiotics and ARG, which are poorly removed during WW treatment (Weng et al., 2022). The *ermB* gene, the most commonly acquired resistance gene, confers resistance to the highly persistent macrolides used in pharmaceutical therapy and livestock and poultry breeding industries (Hua et al., 2019). The *fosA* gene is widely accumulated in aquatic habitats from hospitals and urban WW, and its expression results in the failure of fosfomycin, the first-line therapeutic agent for urinary tract infections worldwide (Güneri et al., 2022). The *qnrS* gene is frequently detected since most WWTPs only remove a small amount of quinolones, turning this resistance into a higher-priority issue due to the significance of these therapeutic compounds in human medicine, particularly in developing countries (Castrignanò et al., 2020). Finally, the *tetA* gene class A (*tetA*(A)) is broadly present in the environment due to the nearly universal use of tetracyclines in livestock production; also, this gene is found in the majority of bacteria isolated from hospital WW (Lépesová et al., 2020).

According to the results of the Kruskal-Wallis and Conover-Iman tests (Figure. 3), the abundances of *aadA*, *ermB* and *tetA*(A) genes were significantly higher in the WWTPs compared to the controls. In contrast, the quantities of the *fosA* gene were significantly higher in both natural environments. Finally, no significant differences were found regarding the abundances of the remaining genetic determinants between WWTPs and the natural samples. Therefore, high quantities of a plethora of ARGs were found in all types of samples from the cold environments analysed here, both in environments subject to significant human pressure and non-anthropogenically disturbed environments. The high prevalence of the genetic determinants of the resistomes of the different cold environments analysed supports the idea that ARGs are ubiquitously distributed in the environment, even in environments with limited human disturbance (such as lava fields and high-mountain lakes), turning the antibiotic resistance in a highly prevalent issue (Liao et al., 2018;Schages et al., 2020).

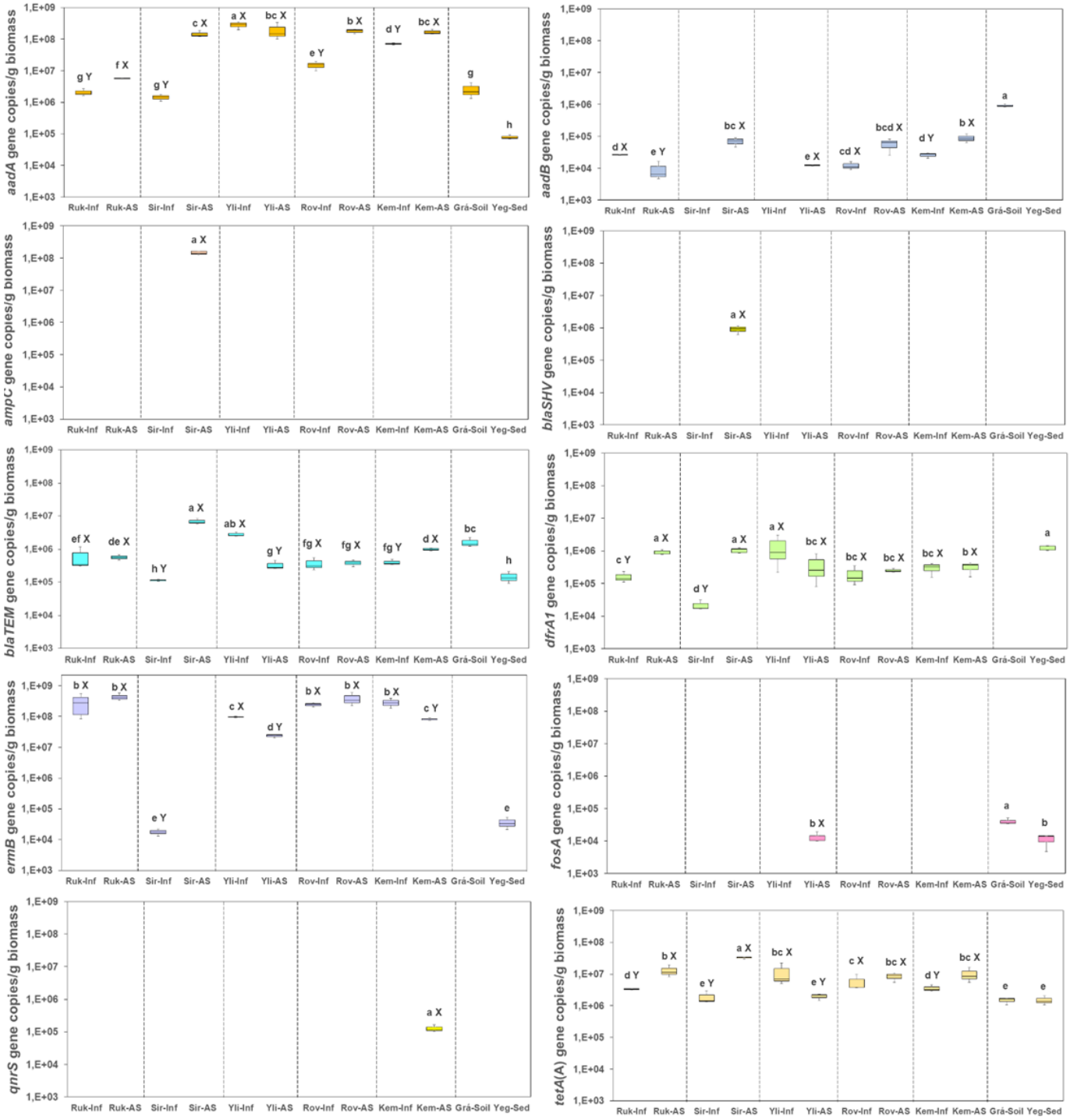

Figure 3. Box–and-Whiskers plots of the genes copies of different ARGs determined by quantitative PCR in samples taken from cold environments (n = 6). Different lowercase letters indicate significant differences among samples, according to the Kruskal-Wallis and Conover-Iman tests ($p < 0.05$). Different capital letters indicate significant differences between influent and activated sludge samples of a given WWTP, according to the Mann-Whitney test ($p < 0.05$).

The abundances of *aadA*, *ermB*, and *tetA*(A) genes were significantly higher in the WWTPs compared to the natural controls (Figure 3). In contrast, the quantities of the *fosA* gene were significantly higher in both natural environments. Finally, no significant differences were found regarding the abundances of the remaining genetic determinants between WWTPs and the natural samples. Therefore, high quantities of a plethora of ARGs were found in all types of samples from the cold environments analysed here, both in environments subject to significant human pressure and non-anthropogenically disturbed environments.

The high prevalence of the genetic determinants of the different cold environments studied supports the idea that ARGs are ubiquitously distributed in the environment, even in environments with limited human disturbance (such as lava fields and high-mountain lakes), turning the antibiotic resistance in a highly prevalent issue (Sukumar et al., 2016; Hao et al., 2021). Generally, the abundances of ARGs analysed were similar between the influent and the activated sludge here, except for *aadA*, *dfrA*1, and *tetA*(A) genes that were more abundant in the activated sludge compared to the influent samples (Figure 3). This could suggest that the secondary treatments were responsible for the removal of *aadA*, *dfrA*1, and *tetA*(A) genes mainly by adsorptive mechanisms, resulting in generated biomasses enriched in these ARGs. This hypothesis has been previously described in other WWTPs, independently of the ARG analysed (Lee et al., 2017; McConnell et al., 2018), making further research necessary to establish the role of the selective removal by adsorption of some specific ARGs compared to the hydrolysis or biodegradation alternatives. In addition, there were distinct patterns of the ARG abundances among the different WWTPs (Figure 3). In agreement with these results, several authors reported that the specific conditions of each WWTP can modulate the bacterial community (Osińska et al., 2016; Ju et al., 2019; Rodriguez-Sanchez et al., 2020; Correa-Galeote et al., 2021; Płaza et al., 2021), which, consequently, results in the broad disparity in the abundance of ARGs among WWTPs.

3.4 Relative abundances of antibiotics resistance genes

To normalise the differences in the total bacterial abundances among samples, the relative abundances of the different ARGs were calculated by dividing the gene copies of a specific ARG by the copy numbers of the 16S rRNA gene (Wang et al., 2016a). The average values of the relative abundances of the ARGs followed the order *aadA* (19.62%), *ermB* (7.22%), *tetA*(A) (3.94%), *dfrA*1 (0.84%), *bla*$_{\text{TEM}}$ (0.58%), *aadB* (0.17%), *ampC*

(0.03%), *fosA* (0.02%), and *qnrS* (< 0.01%) and *bla*$_{SHV}$ (< 0.01%) (Table 1). Similar to the observed total abundances of the ARGs, each WWTP exhibited a proper profile of the relative abundances of the different ARGs. Nevertheless, in general, the high dominance patterns of *aadA*, *ermB*, and *tetA*(A) within the WWTPs were consistent with most compounds used in antibacterial therapy, such as aminoglycosides, macrolides, and tetracyclines, which is in agreement with previously described results (Wang et al., 2016a; Ju et al., 2019; Wang et al., 2020). However, strikingly, a low prevalence of the different types of genes encoding beta-lactam resistance was found in the five WWTPs studied, although beta-lactam resistance genes are prevalent resistance markers in several WWTPs (Yang et al., 2012; Beukers et al., 2018); together with these therapeutic compounds are the most prescribed antibiotics in Finland (Pyörälä et al., 2022). This could be due to the low stability of beta-lactam antibiotics (amoxicillin, AMP, cloxacillin, oxacillin, and penicillin G) at cold temperatures (Riediker et al., 2004), which could result in a lower selective pressure of beta-lactams in the WWTPs minimising the proliferation of ARB resistant to different beta-lactams in these cold environments, according to the strong link between antibiotic concentration and the abundance of the corresponding ARG elsewhere described (Rodriguez-Mozaz et al., 2015; Zhao et al., 2018; Zhang et al., 2022).

The similar dominance pattern between the influent of a given WWTP and the corresponding activated sludge, except for the Sirkka WW treatment facility, should also be noted. This suggests that influent sewage is a primary factor in establishing the resistome within the bacterial communities of the activated sludge of a WWTP. A similar trend has previously been stated in several full-scale WWTPs by analysing the different units of the facilities (Mao et al., 2015; Tong et al., 2019). In general, the values of the relative abundances of the ARGs fell within the ranges of those observed for WWTPs (Laht et al., 2014; Di Cesare et al., 2016; McConnell et al., 2018; Lan et al., 2019; Nguyen et al., 2019; Thakali et al., 2020; Stachurová et al., 2021). Therefore, the cold temperatures at which the different WWTPs operated had no significant detrimental impacts on the quantity of the ARGs in their WW and activated sludge. Besides, it must be mentioned that antibiotic consumption in Finland has been drastically reduced by 25% in recent years (EU/EEA, 2022); however, a reduction in the prevalence of ARGs in the WWTPs has not been observed yet according to the similar values of ARGs compared to other countries with lower reductions in antibiotic consumption. Therefore, the WWTPs

Table 1. Relative abundances of different ARGs (copies ARG/copies bacterial 16S rRNA) determined by quantitative PCR in samples taken from cold environments (n = 6). According to the Kruskal-Wallis and Conover-Iman tests ($p < 0.05$), different lowercase letters indicate significant differences among the gene copies of the different ARGs for a given sample. Ruk-Inf, influent taken from Rukka WWTP, Ruk-AS, activated sludge of Rukka WWTP, Sir-Inf, influent taken from Sirkka WWTP, Sir-AS, activated sludge of Sirkka WWTP, Yli-Inf, influent taken from Ylitornio WWTP, Yli-AS, activated sludge of Ylitornio WWTP, Rov-Inf, influent taken from Rovaniemi WWTP, Rov-AS, activated sludge of Rovaniemi WWTP, Kem-Inf, influent taken from Kemijarvi WWTP, Kem-AS, influent taken from Kemijarvi WWTP, Grá-Soil, Grábrók Volcano soil, Yeguas-Sed, Yeguas Lake sediment, n.d., non-detected (below 10^3 copies/g biomass).

	Ruk-Inf	Ruk-AS	Sir-Inf	Sir-AS	Yli-Inf	Yli-AS	Rov-Inf	Rov-AS	Kem-Inf	Kem-AS	Grá-Soil	Yeg-Sed
aadA	0.081 c	0.120 c	21.631 b	0.297 b	3.047 a	2.273 a	0.354 b	2.233 b	1.057 b	3.988 a	5.679 a	0.771 d
aadB	0.001 f	0.001 f	n.d.	0.001 f	0.001 f	n.d.	0.006 e	0.001 f	0.001 f	0.002 g	2.044 c	n.d.
ampC	n.d.	n.d.	n.d.	0.308 b	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
blaSHV	n.d.	n.d.	n.d.	0.002 e	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
blaTEM	0.024 d	0.013 e	1.707 c	0.014 d	0.031 d	0.004 d	0.009 d	0.005 d	0.006 d	0.023 d	3.676 b	1.446 c
*dfrA*1	0.006 e	0.018 d	0.324 d	0.002 e	0.015 e	0.004 d	0.005 e	0.003 e	0.004 e	0.007 e	n.d.	9.689 b
ermB	24.900 a	20.784 a	0.327 d	17.560 a	1.301	0.337 b	7.297 a	5.975 a	5.260 a	2.417 b	n.d.	0.437 e
fosA	n.d.	n.d.	n.d.	n.d.	n.d.	0.001 e	n.d.	n.d.	n.d.	n.d.	0.089 d	0.109 f
qnrS	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.003 f	n.d.	n.d.
tetA(A)	0.129 b	0.280 b	28.201 a	0.066 c	0.123 c	0.023 c	0.136 c	0.103 c	0.052 e	0.234 c	3.247 b	14.669 a

in cold environments maintain a prominent role as hotspots of ARGs, making mandatory to prevent their dissemination to the fragile and vulnerable receiving ecosystems. In addition, this study highlights the high relative abundance levels of ARGs in the two natural controls. Nevertheless, different dominance patterns were observed between both control samples, with high prevalences of the *aadA*, *aadB*, *bla*$_{TEM}$, and *tetA*(A) genes in the Grábrók Volcano soil, whereas the *tetA*(A) gene dominated the Yeguas Lake sediment. Hence, the analysis of the relative abundances of ARGs reinforced the high prevalence of ARB in cold environments, supporting the assumption that antibiotic resistance is a matter of concern in these cold regions, the single largest biome on Earth.

3.5 Network correlations of the gene copies of bacterial 16S rRNA and ARGs

The analysis of the significant Spearman's rank correlations (ρ>0.29) of bacterial 16S rRNA and ARG total abundances revealed a plethora of statistically significant correlations among total bacterial and ARG-carrying bacterial populations (56.36% of potential correlations; Table 2). Moreover, most of the correlations between genes were positive (47.27%), and there were only four significant negative correlations (pairs *aadA-fosA*, *aadB-dfrA*1, *ermB-fosA*, *fosA-tetA*(A), *fosA-16S rRNA*), accounting for only 9.09% of the possible correlations.

Table 2. Spearman's rank correlation coefficients of the abundances of the bacterial 16S rRNA and the different ARGs in samples taken from cold environments. Negative correlation coefficients are italicized. Absolute values of significant correlations (ρ>0.28) are in bold.

	Bacterial 16S rRNA	*aadA*	*aadB*	*ampC*	*bla*$_{SHV}$	*bla*$_{TEM}$	*dfrA*1	*ermB*	*fosA*	*qnrS*
aadA	**0.763**									
aadB	0.165	0.173								
ampC	**0.473**	0.184	0.260							
bla$_{SHV}$	**0.474**	0.182	0.262	**0.999**						
bla$_{TEM}$	**0.561**	**0.472**	**0.458**	**0.478**	**0.477**					
*dfrA*1	**0.467**	0.229	***-0.326***	**0.335**	**0.337**	0.242				
ermB	**0.642**	**0.332**	0.173	**0.479**	**0.479**	**0.371**	**0.404**			
fosA	***-0.380***	***-0.295***	0.051	*-0.172*	*-0.172*	*-0.139*	*-0.127*	***-0.658***		
qnrS	0.001	**0.291**	**0.315**	*-0.091*	*-0.091*	0.197	0.016	**-0.125**	**-0.172**	
tetA(A)	**0.703**	**0.566**	0.198	**0.477**	**0.478**	**0.564**	**0.450**	**0.755**	**-0.668**	0.235

The corresponding co-occurrence networks were constructed to visualise these significant correlations (Figure 4), showing that all nodes were interconnected, with more pronounced connections for the total abundances of bacterial 16S rRNA, *ermB*, and *tetA*(A). The high level of interconnections among all ARGs analysed, except for *aadB*, *fosA*, and *qnrS* genes, could be related to the fact that the different values of the physicochemical properties were a significant driver in the shaping the ARGs' abundances, in contrast to other factors such as HGT or antibiotic concentration (Forsberg et al., 2014; Su et al., 2015). Henceforth, the shift in the bacterial abundances induced by inherent physicochemical properties is a major driver in the shape of the resistomes, as previously highlighted by analysing the ARGs' abundances of six different full-scale municipal WWTPs (Tong et al., 2019).

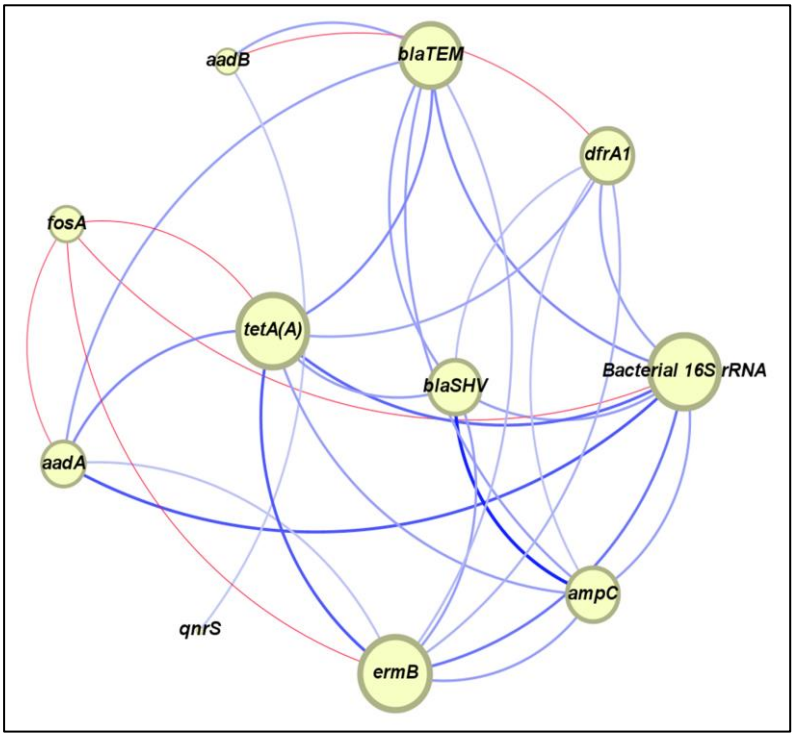

Figure 4. Co-occurrence networks of the significant Spearman's rank correlation coefficients ($p > 0.28$) of the abundances of the bacterial 16S rRNA and the different ARGs (*aadA*, *aadB*, *ampC*, bla_{SHV}, bla_{TEM}, *dfrA*1, *ermB*, *fosA*, *mecA*, *qnrS* and *tetA*(A) genes) in samples taken from cold environments.

On the other hand, the high level of significant correlations among the different ARGs suggests a strong co-occurrence within a given bacterial genome and, subsequently, a common co-selection scenario of genetic determinants encoding antibiotic resistance

proteins among all antibiotic classes (Li et al., 2015c; Pal et al., 2015). Hence, the presence of several multi-drug-resistant bacteria within the bacterial communities of these WWTPs was demonstrated, highlighting these bacterial communities as hotspots for the emergence and dissemination of ARB. Due to the limitation of the qPCR technique in determining the localisation of the different ARGs, it is not possible to determine whether the different ARGs are co-located within the same pathogenic island or not placed adjacently in their genome. Therefore, further research is necessary to analyse the genetic structure of the pathogenicity islands in the ARB of the cryosphere to establish the importance of multi-drug resistant bacteria within these bacterial communities.

4. CONCLUSIONS

This study addressed the occurrence of several important ARGs from samples taken in different cold environments, including five WWTPs and two non-disturbed natural controls. For that purpose, the abundances of *aadA*, *aadB*, *ampC*, *bla*$_{SHV}$, *bla*$_{TEM}$, *dfrA*1, *ermB*, *fosA*, *mecA*, *qnrS*, and *tetA*(A) genes were determined using a recently developed qPCR methodology that allows the more accurate quantification of the bacterial populations carrying these genes. The analysis of the ARGs' quantification showed a high ARG abundance in the cryosphere samples, irrespective of the sample type, except for the hospital-related *mecA* gene, which was not present in any of the samples analysed. Notably, the *aadA*, *ermB*, and *tetA*(A) genes were the most prevalent ARGs in the WWTP samples. Except for the Sirkka WWTPs, a similar dominance pattern of the relative abundances of the ARGs was found between the influent and activated sludge sample of a given WWTP, highlighting that the influent sewage is a primary factor in the establishment of the resistome of the corresponding activated sludge. In addition, the high abundances of the ARGs in the influent samples resulted in strong ARB prevalences in the activated sludge. In this context, WWTPs in the cryosphere are hotspots in disseminating multi-drug ARB to the receiving water bodies, despite the selection of the resident microorganisms by cold temperatures. Finally, although with their own ARG profiles, the samples taken from cold environments with low anthropogenic activity also had high levels of ARGs, suggesting that antibiotic resistance is ubiquitous and highly present in the cryosphere, the single largest biome on Earth. This calls for the importance to monitor and control the presence of ARGs in WWTPs in cold areas to prevent the dissemination and proliferation of ARB to the receiving ecosystems.

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Author contribution

J. González-López and A. González-Martinez devised the project and the funding acquisition; L. Perez-Bou conducted experiment and contributed to data analyses, B. Muñoz-Palazon performed samplings; D. Correa-Galeote conducted methodology, data analyses, devised to the manuscript's conception and design and wrote the manuscript. All authors read and approved the final manuscript.

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Conflict of Interest

The authors declare no competing interest

5

5-GENERAL DISCUSSION

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Hospital WW is a complex matrix comprising a wide range of toxic environmental pollutants (Bhandari et al., 2023) that poses a potent challenge to the security of human and environmental health (Kumari et al., 2020). Chemical and microbiological characterization of hospital effluents have reflected a failing quality and reinforce the high harmful and susceptibility to the outbreak of several waterborne diseases (Carraro et al., 2016; Majumder et al., 2021; Fatimazahra et al., 2023). The high flow rates of BOD_5, COD, ammonia, and nitrogen determine their low biodegradability index, making them difficult to treat by CAS in WWTPs (Verlicchi et al., 2015). Besides, there are other several relevant pollutants such as PhACs, ARB and ARGs in these effluents, persisting after CAS treatment. Although CAS remains the most widely used biotechnology for WW treatment, the low capacity for PhACs removal requests to search for better alternatives. In this regard, several studies have suggested that the biological processes performance may be more suitable by advanced treatment strategies due to the metabolic diversity of microbial communities of these sludge and, also, by their resistance to the high flow rates and the low energy and cost demand associated to them (Oberoi et al., 2019; Zhu et al., 2021; Langbehn et al., 2021; Bhandari et al., 2023).

AGS technology has emerged as a sustainable WW treatment for high polluted WW treatment (Nancharaiah and Reddy, 2018; Cui et al., 2021). The smaller size, operation in a single unit, greater adaptability to treat fluctuating loads of contaminants, more compact granules with high settling rates, higher retention of metabolically active biomass and less sludge residue improve the overall performance of AGS compared to CAS (Vydehi et al., 2024). However, extremely limited research addressing the effectiveness of AGS systems to treat hospital WW treatment has been made, mostly using synthetic WW (Kang et al., 2018 a,b; Cui et al., 2021; He et al., 2021). Thus, there are a plethora of gaps in the performance of AGS treating hospital WW, which has served as the motivation for the development of the current investigation. Therefore, it is necessary to thoroughly investigate the effect of the complexity of real hospital WW, with the ample and changing presence of PhACs, on the granular biomass performance. In ***Chapter I***, a laboratory-scale AGS was operated for 150 days with raw hospital WW and periodically monitored for nutrient and PhACs removal rates, granule stability, abundance of key microbial populations and microbial diversity. Although the hospital WW characteristics varied

over the operating period, the main physicochemical parameters (nitrogen, phosphorus, COD and BOD_5 removal and settling velocity) achieved adequate removal rates, meeting the requirements of the European Union (Directive 2000/60/EC). It should be noted that, some of the most attractive advantages of AGS stood out under real conditions, i.e., high toxicity resistance, broad versatility, granular biomass stability (Amorim et al., 2018; Li et al., 2020 a,b; He et al., 2021), as it was shown in ***Chapter I***, reflecting the advantages of the use of AGS technology to treat complex influents.

In general, it is common for biological systems to require more extended periods of acclimation when the environment is significantly different to this of the initial inoculum (Franca et al., 2017; Muñoz-Palazon et al., 2022a). This trend was observed in ***Chapter I***, due to the occurrence of two phases during the experiment. For the first 37 days, an adaptation phase was required, corresponding to the expected effects of complex hospital WW on the physical and functional stability of the granular biomass. The second period (from day 38 to day 150) was characterised by the stability of the COD and BOD_5 removals, which were more than 75% and almost 100%, respectively. Thus, as reflected by this steady-state trend, the previous adaptation phase of the granular biomass to the hospital WW promoted an excellent efficiency in terms of organic matter removal. Results in agreement with those of Muñoz-Palazon et al. (2021), who informed the existence of two periods during the 180 operational days of the AGS system inoculated with mature granules treating synthetic WW containing PhACs, which shows a decreasing trend in nutrient removal and a strong depletion of MLSS in the system during the start-up phase, but eventually removal rates were higher up to 80% in the steady state scenario. Summarizing the results obtained in ***Chapter I***, the long-term operation of the AGS in terms of organic matter degradation is feasible when real hospital WW was employed, as it was demonstrated when domestic (Ab Halim et al., 2016) and synthetic hospital WW was employed (Muñoz-Palazon et al., 2022a).

On the other hand, the enhanced nitrogen removal rate is another key operational parameter of AGS reactors, given the benefits of nitrification and denitrification processes at the same time, which is facilitated by the stratification of the microbial populations within the granular sludge (Gonzalez-Martinez et al., 2016; Rodriguez-Sanchez et al., 2016). In ***Chapter I***, total nitrogen removal values were close to 70%, and ammonium oxidation values were close to 90%. In addition, the stable prevalence of microorganisms

involved in ammonia oxidation and nitrate denitrification was verified by absolute quantification of *amoA* and *nosZ* genes, respectively, as it was previously reported in other AGS operations under a strong PhACs influence (Muñoz-Palazon et al., 2021 b; Muñoz-Palazon et al., 2022a). Thus, the nitrogen removal was successfully achieved by the operated AGS under the conditions employed here, confirming the robustness of the AGS technology to remove nitrogen.

In terms of phosphorus removal, as seen in ***Chapter I***, the phosphate removal rates were not competitive at start-up and reached a maximum performance of 50% in the steady state. Usually, PAOs are located in granular anaerobic interlayers performing the biological phosphorus accumulation process (Rosa-Masegosa et al., 2021). Comparing the results of this thesis, the values obtained here are satisfactory, since the phosphate removal rates achieved by other AGS systems without PhACs addition have been informed in the low range of 25-35% (Gonzalez-Martinez et al., 2017) to 50-60% (Gonzalez-Martinez et al., 2018).

The feasibility of AGS in terms of mean particle size and settling velocity stability was also addressed in ***Chapter I***. The stability of AGS was considered to not present significant variation in mean granule size, although the diameter decreased from 4.0 mm at the inoculum to 2.0 mm at the end of the operation, as well as no occurrence of granule disintegration and washout from the reactor was observed. This resulted in granules less compact and smoother than those used as inoculum, with a progressive decrease in settling rate. AGS typically has a particle size ranging from 0.3 mm to 10.0 mm in diameter (Pruba et al., 2020; Han et al., 2022, Liu et al., 2023). Granular size has a strong influence not only on the settling capacity but also on the mass transfer (Long et al., 2019; Muñoz-Palazon et al., 2022a). Larger particle sizes have greater mass and less resistance to settling in the water, but excessively large particles can hinder nutrient transfer and the growth of filamentous microorganisms (Hou et al., 2021). In contrast, smaller sizes have a larger surface area relative to their volume, which can improve the overall performance of the system (Shameem and Sabumon, 2023). It has been highlighted that more compact granules tend to settle faster due to the greater gravitational force acting on them and less prone to disintegration (Hamza et al., 2021; Hou et al., 2021; Ekholm et al., 2022; Lin et al., 2023) and that the increase in the mean size and compactness during the operation time is an indicative sign of its promoted functionality and stability (Gonzalez- Martinez

et al., 2017, Hurtado-Martinez et al., 2021; Muñoz-Palazon et al., 2022a). Generally considered, the PhACs can promote granule breakage and disrupt the metabolically functional microbial structure involved in AGS performance (Cui et al., 2021; Zheng et al., 2024). For example, Amorin et al. (2016) reported a temporal affection AGS morphology related to a CIP exposure, which resulted in a decrease in granular size, accompanied by an increase in biomass washout and deterioration of the sludge's settling properties. Strikingly, in this study, despite the changes in the physical properties of the granules, no significant variation in metabolic activity in terms of COD, BOD_5, nitrogen and phosphorus removal performances of the reactor was observed. In agreement with this, other studies have reported that the average size of the granules could be even larger and more compact in AGS treating drugs than those of AGS without PhACs addition (Rodriguez-Sanchez et al., 2017; Wang et al., 2018 b; Muñoz-Palazon et al., 2022a).

The proper treatment of complex WW with a high load of PhACs by AGS reactor has been confirmed in the last decades (Amorim et al., 2014; Muñoz-Palazon et al., 2021b). The ability of AGS to significantly reduce high levels of PhACs from real hospital WW without compromising overall performance was demonstrated in this study (***Chapter I*** and ***Chapter III***). Based on the clinical relevance and widespread environmental prevalence of PhACs (Amorim et al., 2014; Muñoz-Palazon et al., 2021b), a total of seventeen different compounds were monitored in raw influent and final effluent samples over time. In this regard, CMP (antidepressant); CCLP and TRC (antitumoral); KTP, DCL, and NPX (anti-inflammatories) and the antibiotic compounds GEN (aminoglycoside); AMP and amoxicillin (beta-lactams); AZN, CLM and ERY (macrolides); DOX (tetracyclines); SMX (sulfonamide); CIP (quinolones) and TMP (pyrimidine) were quantified. A high variability of PhACs concentrations in the influent water was observed over time. Significantly, the highest drug loads (TMP, CMP, NPX and DCL) were detected on day 60, defining two phases in the operation of the AGS system. In general, high removal efficiencies (>70%) of target PhACs from the different therapeutic classes were achieved, except for TRC, KTP and AMP which have elimination rates of around only 50%. Notably, TMP, CMP, and NPX, which were highlighted as some of the most recalcitrant PhACs in previous AGS systems (Rodriguez-Sanchez et al. 2017; Muñoz-Palazon et al. 2021, 2022) and CAS systems (Radjenovic et al., 2007; Oluwole et al., 2020), were successfully removed in the AGS reactor feeding with real hospital WW. Finally, despite the active release of some PhACs from the AGS

system in certain periods, the technology is still postulated as a competent technology for the removal of PhACs, confirming the results of Rueda-Márquez et al. (2021).

The diversity of microbial populations in AGS systems can be significantly affected by the presence of PhACs, exerting selective pressure that can lead to the dominance of certain resistant species while reducing overall diversity (Ren et al., 2023). Despite changes in microbial community composition, AGS systems often exhibit functional redundancy, meaning that different microbial species can perform similar ecological roles, maintaining system stability even when PhACs impact negatively on specific populations (Louca et al., 2018; Xia et al., 2018). In this manner, in ***Chapter I*** the temporally changes in the microbial composition of AGS treating hospital WW were analysed. In terms of abundance, the results indicated that the high loads of PhACs affected the abundance of microbial populations. Notably, this effect was even stronger in the fungal and PAO communities, even though both communities are found in the interlayer zones and core, respectively of the granule (Muñoz-Palazon et al., 2020 a; Nancharaiah and Kiran Kumar Reddy, 2018). The layered structure of AGS allows the retention of harmful compounds from the outer layers to the inner layers, acting as a protective barrier to microorganisms residing in the core. However, over time, their accumulation can cause progressive deterioration of the granules, even leading to the depletion of key populations in the inner layers, especially in long-term operations (Rosa-Masegosa et al., 2021; Liu et al., 2023). In this study, bacterial and fungal communities acclimated to pharmaceutical input satisfactorily, while archaeal populations adapted progressively over time. Thus, the higher COD and BOD_5 removal rates were strongly and positively correlated with the abundances of *Bacteria*, *Archaea*, *Fungi* populations and denitrifiers, suggesting a functionally stable core microbiome able to resist the fluctuating composition of water inlet over time.

The most dominant phylotypes of the Archaea community in the granules employed as inoculum were *Bathyarchaeota* phylum and *Methanocaldococcus* genus members. In terms of diversity and evenness, α diversity indices indicated a similar structure of the archaeal communities from day 0 until the end of the experiment, while species richness (Chao index) increased over time from 60 ± 14 to 173 ± 3. Also, the role played by *Methanocaldococcus* was reinforced by reaching 50 % of the total relative abundance from day 30 until the end of the operation. This genus could play a leading role in organic matter removal via methanogenesis reactions and sludge stabilisation, which is a

functionality confirmed in anaerobic digestion and CAS systems. (Yan et al., 2019). The role of *Bathyarchaeota* could be associated with their great metabolic diversity that supports the growth of other beneficial microorganisms, such as methanogens and ammonia-oxidizing archaea through syntrophic relationships (Xiang et al., 2017; Zhou et al., 2018; Yan et al., 2024). Interestingly, in this study, *Bathyarchaeota* was correlated with high TMP and DCL removal rates, whereas *Methanocaldococcus* genus was strongly and positively correlated with the KTP, TRC, nitrogen and phosphate removal rates. The metabolic versatility of *Archaea* is generally more limited (Chen et al., 2018). However, these results support the critical role of archaeal communities in the functional performance of the bioprocesses for hospital WW treatment, first described in this study.

Concerning fungal populations, a specialisation of the community was evidenced during the operation time, suggesting a lower metabolic versatility of this community to cope with the effects of the broad presence of PhACs. Nevertheless, the structure of the fungal communities changed at day 60, after a high exposure of PhACs, suggesting that the dynamic of fungal communities is critical in restoring physical granule biomass properties and metabolic relationships for nutrient removal after a high load of PhACs. Some of the most abundant and ubiquitous members of the fungal populations, *Nectriaceae* until day 60 and *Trichosporonaceae* after day 60 are highlighted to play alternatively essential roles in granule nucleation and syntrophic relationships for the degradation of organic compounds. Such functions and capabilities were confirmed by the RDA of fungal communities when 23 OTUs were positively correlated with high drug removal efficiency throughout the experimental period.

In general, bacterial population dynamics revealed a great diversity and functional essential role for AGS performance. A large number of dominant bacterial OTUs (69) were correlated with a good performance in terms of TMP, NPX, CCLP, BOD_5, NH_4^+, PO_4^{3-} and N removals. The dominant phylotypes *Dokdonella*, unclassified *Comamonadaceae*, and *Hyphomicrobium*, according to their high abundances, dominant pattern and versatile metabolic functions previously reported in CAS and AGS systems (Palma et al., 2018; Muñoz-Palazon et al., 2021; Chen et al., 2023), played critical role in the removal of toxic and complex organic compounds and in ensuring the granular biomass properties. Regarding the dynamics of the bacterial communities, different studies have shown that the presence of PhACs can affect the microbial community, leading to changes in the dominant bacterial species (Langbehn et al., 2021; Khalidi-

Idrissi et al., 2023). In this trend, *Dokdonella* and *Hyphomicrobium* were deeply depressed after day 60, while *Saprospiraceae* and *Xanthomonadacea* family members increased significantly their relative abundance, showing great resilience and adaptability of the whole bacterial community established within the granule. The family *Saprospiraceae* could contribute to the structural integrity and functional stability of the AGS performance due to their ability to degrade complex organic matter, including proteins and polysaccharides, as well as recalcitrant compounds, which are often in hospital WW (Zheng et al., 2024; Lou et al., 2024). Bacteria belonging to the *Xanthomonadaceae* family have a wide range of metabolic capabilities and can adapt to different environmental conditions. In bioprocesses for WW treatment, such as MBR and constructed wetlands, these bacteria can play an important role due to their ability to break down various organic compounds, including PhACs and other contaminants commonly found in hospital WW (Zhao et al., 2022; Mumtaj et al., 2024).

The evolution of bacterial communities resulted in the shaping of the abundance of ARGs and prevalence of ARB, which vary about the chemical composition of the WW, the operating conditions and the selective pressures of harmful compounds (Chen et al., 2016, Xia et al., 2018; Li et al., 2020 a,b; Adler and Holliger, 2020). Given the acceleration of antibiotic resistance emergence as a critical public and environmental health concern is mandatory to limit the impact of antimicrobial resistance is essential today (Goldmann et al., 2024). Therefore, understanding the dynamics of ARGs in biological systems may help develop strategies to mitigate their proliferation and spread by optimizing operating conditions and integrating additional treatment technologies to reduce ARG levels. This type of quantitative study to determine the abundance of AGRs in granular biomass was supported by a new qPCR methodology developed in ***Chapter II***. Thus, this thesis implemented a new set of primer and the corresponding qPCR protocols to quantified eleven ARGs including the genes *aadA* and *aadB* (aminoglycoside resistance), *ampC*, *bla*$_{SHV}$, *bla*$_{TEM}$, and *mecA* (beta-lactam resistance), *dfrA*1 (TMP resistance), *ermB* (macrolide resistance), *fosA* (fosfomycin resistance), *qnrS* (quinolone resistance) and *tetA*(A) (tetracycline resistance), which were selected due to their clinical importance and their high prevalence in WWTPs and receiving aquatic ecosystems (Wang et al., 2020; Nguyen et al., 2021). The design and validation of the new molecular tools accomplished the quality properties suggested in reference publications (Bustin and Huggett, 2017; Deider et al., 2020). In this regard, several key parameters such as target selection, high

orthology (>70%) to cover the biodiversity of the ARGs, optimal melting temperatures, proper GC content (<50%), and lack of secondary structures were checked using *in silico* PCR tools to guarantee the quality of primers design in terms of specificity and total coverage. Besides, complete coverage of the whole biodiversity of clinically and environmentally relevant ARGs in currently available databases was ensured. Validation studies were then conducted using natural and engineered environments related to WW treatment.

For the *in vivo* validation studies, sludge-forming biological processes such as CAS, composting and anaerobic digestion were used to assess the abundance of target ARGs in relation to their abundance in receiving natural ecosystems (agricultural soil and Genil River sediments from Granada/Spain). In general, a high detection frequency of ARGs (ranging from 70 to 100%) was found in all the samples. The most prevalent genes detected were *aadA*, *aadB*, *bla*$_{\text{TEM}}$, *dfrA*1, and *fosA* genes, whereas *ampC*, *bla*$_{\text{SHV}}$, *ermB*, *qnrS*, and *tetA*(A). Among them, *aadA* (average 2.81×10^{7} copies/g) and *ermB* (average 2.75×10^{6} copies/g) were the most abundant genes in all the samples. Notably, the *mecA* gene was not detected in any samples. The absence of the *mecA* gene in the environmental samples is consistent with the previous report by Shoaib et al. (2020), although methicillin-resistant strains have been increasingly detected in recent years, mainly in WWTPs (Oladipo et al., 2023; Philo et al., 2024) and hospital-associated environments (Mehanni et al., 2023). The natural environment showed a distinct profile of ARGs, as evidenced by the non-detection of *ampC*, *bla*$_{\text{SHV}}$, *ermB*, *qnrS* genes. This study snapchats the content of ARGs of several bioprocesses for WW treatment and highlights the widespread occurrence of ARGs targeting the most commonly used antibiotics. As a main result, the qPCR assays and the new primer sets were effectively validated and disclosed as potentially useful to monitor antibiotic resistance and especially revealed the role of WW-related bioprocesses as a hotspot of ARGs.

Chapters III and ***IV*** implemented these new qPCR protocols to address the profile of ARGs in the AGS reactor treating real hospital WW. Hence, ***Chapter III*** determines the abundance of target ARGs in the granular biomass under the occurrence of antibiotic compounds. Despite the sustainable performance of AGS systems treating hospital WW, an undeniable enrichment of ARGs in the granular biomass was demonstrated. The most abundant gene in the granular biomass was *aadA* (average 3.16×10^{8} copies/g), followed by *aadB* (8.28×10^{7} copies/g), *ermB* (1.94×10^{7} copies/g), *dfrA*1 (1.26×10^{7} copies/g),

qnrS (1.18×10^7 copies/g) and *tetA*(A) (9.32×10^7 copies/g) genes which show middle prevalence. A low abundance of bla_{TEM}, bla_{SHV} and *ampC* (in average values, 2.42×10^6), suggested a no prevalence among ARB dominant in AGS biomass. Notably, the detection of the *fosA* gene showed the lowest abundance and the hospital-related *mecA* gene was not detected in any of the sampling days. The prevalence of the *aadA*, *aadB*, bla_{SHV}, *dfrA*1, *ermB*, *fosA*, *qnrS*, and *tetA* (A) genes exhibited a notable increase from day 30 until the end of the experiment, indicating an enrichment of these genes in granular biomass. The accumulation in sludge biomass of ARGs in different lab-scale WW treatment systems has previously been confirmed (Zhang et al., 2013; Zhu et al., 2018; Gao et al., 2018; Li et al., 2020 b). These results can be related to the influence of the raw hospital WW due to the differences in the epidemiology of ARB in hospital settings (Hocquet et al., 2016), the operating conditions of the AGS system (Zhang et al., 2019 b; Li et al., 2020 a,b), the adaptation/restructuring of the bacterial community in granular biomass (Amorin et al., 2014), the selecting power of antibiotics on ARGs prevalence and, finally, the co-selection phenomenon of non-corresponding ARGs (Zhang et al., 2019b, Li et al., 2020 a,b).

According to this ARG enrichment in the granular biomass, it will be essential to limit the accumulation of ARGs in sludge, which has been strongly avoided in current implementations. As a strategy to limit the proliferation of ARBs and increase the removal rates of antimicrobial compounds, a reduction in the exposure time through the optimization of HRT and SRT parameters has been proposed as key factors to achieve these goals (Kang et al., 2018b; Wang et al., 2022). Also, both parameters have significant impacts on the growth of filamentous bacteria, which can affect the overall performance of the system. A well-balanced HRT and SRT ensures sufficient contact time for biodegradation and allows the growth of slow-growing microorganisms that are crucial for the degradation of complex organic compounds (Li et al., 2013; Guven et al., 2017, Wang et al., 2021). Nevertheless, the impact of these parameters on the fate of ARGs in AGS systems represents a crucial area of research in the field of WW treatment scarcely addressed. Therefore, as concluding remarks, longer retention (HRT and SRT) facilitates microbial degradation processes; however, it may also potentially increase the frequency of HGT (Rosman et al., 2014; Liu et al, 2016; Liao et al., 2019). Thus, the optimization of AGS technology in terms of proper retention times needs to be conducted, especially when real hospital WW is treated.

A recent study addressing different-sized granular biomass under antibiotic pressure highlighted that moderate granule size (1.0 to 2.0 mm) tend to have the lowest ARG abundances compared to small and large granules due to the highest protein level in the EPS layer, which enhances mass transfer resistance and effectively reduces antibiotic stress on bacteria (Liu et al., 2024). Hence, an alternative strategy to reduce the enrichment of AGS is controlling the size of the granular biomass, which opens up new ways of dealing with the abundance of ARGs in the AGS, which is crucial for reducing the prevalence of ARGs in WW treatment applications as a useful strategy to reduce the risk of antimicrobial resistance.

While extensive research has delved into AGS to explore aspects such as physicochemical performance and diverse applications, the current investigations of ARG prevalence have been neglected. Nevertheless, much more research has been conducted on traditional flocculent sludge (Hu et al., 2024). In this regard, Alexander et al. (2021) reported a large contribution of 23 different WWTP effluents in Germany to the spread of 12 ARGs and associated pathogenic bacteria, releasing approximately 10^9 to 10^{10} ARGs equivalents per day. Independently of the scarce research on ARGs dynamics and fate in AGS systems, AGS systems typically exhibit low or null production of waste biomass in relation to CAS (Lv et al., 2014; Abbass et al., 2018), decreasing the amount of potentially harmful biomass enriched in ARGs and, subsequently, improving the role of AGS systems compared to that of CAS to reduce the contribution of the generated sludge to the antibiotic resistance issue. However, an AGS treating SMX-enriched synthetic WW showed an increase in floccular biomass content and a massive loss of incompact sludge fraction with higher content of ARGs (multi-drug resistance, aminoglycoside, and tetracycline) than mature granular biofilm (Ren et al., 2023). Thus, further studies need to be conducted to provide a more comprehensive understanding of the prevalence and spread of ARGs during AGS treatment processes.

According to the aforementioned facts, WWTPs are the leading players in the dissemination of the antibiotic-resistance phenomenon, highlighting the necessity of determine the prevalence of antibiotics and ARB by continuously monitoring the occurrence and abundance of ARGs (Kena et al., 2020), especially in habitats that are highly vulnerable to anthropogenic impacts, to protect them from environmental contamination and the harmful effect of the discharge of WW effluent without a proper removal of antibiotics and ARGs in them. Among these habitats, cold environments stand

out due to their particularly sensitive nature to changes and their essential role in global environmental stability. Cold-temperature habitats are the most widespread extreme territories in the world, housing a wide diversity of unique species adapted to extreme conditions. In particular, psychrophilic bacteria drive critical global biogeochemical cycles and are the most abundant, diverse and widespread extremophiles (Sayed et al., 2020; Shen et al., 2021). Despite few studies having quantified the occurrence and abundance of ARGs and host psychrophilic bacteria, particularly in WWTPs located at low temperatures (Sun et al., 2021; Liang et al., 2023) their role as hotspots for the spread of ARBs and ARGs need to be thoroughly investigated.

Thus, ***Chapter IV*** dealt with the resistome of five WWTPs located in cold northern areas with mean annual temperatures below 5 °C, using the new qPCR methodology developed in this thesis. In particular, the abundance of target ARGs in psychrophilic bacterial communities from activated sludge samples and the contribution of raw sewage influent to their prevalence were determined. The quantitative PCR studies of ARGs showed a strong occurrence and abundance of these genetic determinants in natural and WW treatment cold-related environments. In this way, the CAS and MBR biological treatment processes implemented in those WWTPs constituted important potential reservoirs of ARB. Also, the soil and sediment samples from two undisturbed cold environments showed a similar trend. Nevertheless, the *mecA* gene was not detected in any of the samples. *mecA* gene is mainly prevalent in hospital-associated environments due to the prevalence of methicillin-resistant *Staphylococcus mecA*-positive strains, which resist almost all types of beta-lactams (Mehanni et al., 2023). Surprisingly, it was not detected either in the hospital WW nor in any environmental sample. In contrast, prevalence studies in WWTPs located around the world detected a significant abundance of *mecA* gene. Notably, 77% of the staphylococcal isolates in WWTPs located in South Africa harbour *mecA* gene (Oladipo et al., 2023). Similarly, WWTPs from Portugal, King County, and the United States reported a significant increase in the prevalence of the *mecA* gene in WW samples in the last years (Philo et al., 2024). By the similar distribution pattern of ARGs in different WWTPs from global regions (Rodríguez et al., 2021, Liang et al., 2023), the most abundant genes were *aadA*, *ermB* and *tetA*(A). It must be noted, the significative contribution of raw water on the abundance and prevalence of the 10 ARGs detected in the biomasses of the cold-related WWTPs here employed, as it

has been informed in several activated sludge samples from WWTPs along the world (Wang et al., 2020; Li et al., 2020c; Shi et al., 2021).

In addition, this study revealed a high level of significant positive correlations between the different ARGs, suggesting strong co-occurrence and potential co-selection events of ARGs encoding resistance to different antibiotic classes, and reinforcing their prevalence in granular biomass (***Chapter III***) and activated sludge samples (***Chapter IV***). In particular, the occurrence of antibiotics may play a significant role in the prevalence of ARGs, which was demonstrated when a plethora of strong positive correlations were observed for some antibiotics and the ARGs, including those that confer antibiotic resistance to them, such as CIP-*qnrS*, ERY-*ermB*, GEN-*aadA* and *aadB* (***Chapter III***). Interestingly, non-corresponding antibiotics could also have a strong selection effect on the prevalence of ARGs, which have been previously reported in AGS reactors (Zhang et al., 2019 b; Hu et al., 2024). This fact is relevant because of the potentially high frequency of HGT via plasmid-mediated resistance carrying the multidrug resistance determinants (Liu et al., 2019 a). These transfer events favor the presence of the antibiotic and other non-antibiotic chemical compounds often detected in the WW inlet, which provides a favorable background for the selection and proliferation of other additional resistance mechanisms (Shi et al., 2022). In this regard, the high abundance of the ARGs in the influent samples resulted in strong ARB prevalences in the activated sludge (***Chapter IV***), that WWTPs are hotspots for the proliferation of multi-drug ARB. In addition to the influence of the incoming water, several studies also described the influence of specific characteristics of the WWTPs related to physicochemical, environmental, and even climatological variables, on the abundance of ARGs in different regions at a global level (Hong et al., 2013; Novo et al., 2013, Rodríguez et al., 2021).

To summarize the approaches of quantitative studies (***Chapter II***, ***Chapter III***, and ***Chapter IV***), the results highlighted the high prevalence of the target ARGs in natural and engineered environments related to WW treatment. In general, the *aadA*, *aadB*, *bla*$_{\text{TEM}}$, *dfrA*1, *ermB* and *tetA*(A) genes were the most prevalent, which is to say, these genes were found in the majority of the sampling times analysed, highlighting that these genes are essential pieces of the complex puzzle of antibiotic resistance in WWTPs. The *ampC*, *bla*$_{\text{SHV}}$ and *qnrS* genes were not detected in any natural samples i.e., sediment (Genil River and Yeguas Lake), and soils (volcano and agriculture soil), showcasing the necessity to reduce the amounts of beta-lactams and quinolones arriving to the

environments to maintain the antibiotic resistance to these groups in low levels, ensuring the therapeutic effects of these essential PhACs. Remarkably, the null occurrence of *mecA* in the samples, including both natural and engineered environments associated with WW treatment, emerges as an environmental and clinical advantage, demonstrating the acute need to continue to confine the treatment of methicillin-resistant *S. aureus* to healthcare facilities.

The total abundances of the different ARGs among all samples oscillated from below the limit of quantification ($<10^3$ copies/g of biomass) to 10^9 copies/g of biomass. The most abundant was the *aadA* gene (in average values $6{,}43 \times 10^9$ copies/g of biomass), followed by decreasing abundance values of *ermB* and *dfrA*1 genes (10^8 copies/g of biomass). The abundance of the remaining genes was in the order of 10^7 copies/g biomass, with the *fosA* gene showing the lowest abundance in the full set of samples (105 copies/g biomass). These results are consistent with the dominance patterns of those ARGs in different environments such as WWTPs (Wen et al., 2016; Quintela-Baluja et al., 2019), soils (Zhang et al., 2017 b; Liu et al., 2022) and sediments (Li et al., 2018; Wang et al., 2021), and, also, with their use as the most commonly prescribed antibiotics worldwide (beta-lactam, aminoglycosides, sulfonamides, macrolides, and tetracyclines) (Wang et al., 2016 b; Ju et al., 2019; Wang et al., 2020). Decisively, this study supports the evidence that the global spread of ARGs and ARB is a growing phenomenon, in engineered environments related to WW treatment and even in pristine environments.

Some slight differences in the resistome profiles were found among the samples used in this thesis and other prospective studies. These findings may be related to the differing epidemiology and regionally varying prevalence of ARBs in hospitals (Hocquet et al., 2016). Nevertheless, prevalence studies to determine the epidemiological distribution pattern of clinically relevant genes are still mandatory to prevent the transmission and evolution of infectious diseases, in particular, monitoring of ARGs related to the most relevant pathogens from primary sources (WWTPs) could contribute to achieve this goal (Grad et al., 2014). In this regard, recently, it has highlighted WW based epidemiology, encompassing the monitoring of antibiotic content and determine the “fingerprint” of the resistome of pathogenic bacteria that host different ARGs in the different compartments of the integral WW treatment cycle, as an efficient alternative to track antibiotic resistance and the abuse of antibiotic consumption (Picó and Barceló, 2021). Besides, under the concept of “One Health”, an integrated, unifying approach that aims to sustainably

balance and optimise the health of people, animals and ecosystems, it is necessary to transfer these actions to national and regional levels to combat the antibiotic emergency by the United Nations Environment Programme (WHO, 2022). In this concept of recognising that the health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and interdependent, the monitoring of antibiotics, ARB and ARGs should be essentially taken in the political implementations of the antibiotic resistance issue to generate sufficient information to establish future regulations aimed at reducing the proliferation of the most worrying pathogens in our society, while ensuring the effectiveness of antibiotics against future generations. To date, in Spain and locally in the province of Granada, no systematic studies have been carried out to evaluate the phenomenon of antibiotic resistance in environmental samples under the triple prism of human health, animal health and the environment. This thesis is pioneering the research based on WW-based epidemiology in the antibiotic, ARB and ARGs fields by analysing different environmental compartments to provide a global snapshot of the antibiotic resistance in Granada.

Finally, this study was the first report of the feasibility of the AGS technology to treat in situ real hospital WW. In general, the AGS reactor operated achieved high nutrient and PhACs removal rates, accurate granular biomass characteristics, and metabolic and functional stability of key microbial populations under the effect of variable loads of antibiotics in the influent. Once again, WW treatment based on AGS systems emerges as a reliable and highly efficient bioprocess, helping to meet the current global challenge of WW treatment, especially for *in situ* hospital WW treatment alternatives. However, a significant increase in the abundance of ARGs in granular biomass was observed, reinforcing the urgency of carefully monitoring the contribution of AGS to proliferation and spread in further studies. To limit these gaps, the AGS technology must be optimised in terms of technical and health guarantees.

6

6-GENERAL CONCLUSIONS

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1. The AGS technology has high capabilities to treat real hospital WW, highlighting the high stability of the granular biomass and an excellent performance in the removal of organic matter and nutrients. Notably, the removal of common pollutants and clinically relevant drugs was successfully achieved, particularly for some of the most well-known recalcitrant compounds (trimethoprim, carbamazepine and naproxen). Therefore, the AGS is a suitable technology to efficiently treat hospital WW before discharge into the sewage network systems.

2. Antimicrobials and toxic compounds found in the raw hospital WW had a broad impact on the microbial population dynamics of the bioreactor, leading to a progressive adaptation and restructuring of the bacterial, fungal and archaeal communities. Despite of this, the global performance of the AGS reactor remained steady, as the microbiome developed in the granular biomass had remarkable resilience under the fluctuating composition and concentrations of PhACs in the raw WW, ensuring the metabolic and structural functions in granular biomass over the operation time.

3. The new quantitative PCR methodology developed here is a valuable tool to monitor the abundance and distribution of the target ARGs in relevant natural, anthropogenic, and healthcare environments, generating reliable information and avoiding the underestimation of the ARGs' biodiversity. The use of the new primer sets revealed the prevalence of the most important clinical and environmental ARGs in the WW-related environments, indicating the urgent requirement to transform WWTPs from hotspots in the generation and dissemination of ARB into a sink of them before the discharge of WW into receiving waterbodies.

4. The dynamics of the ARGs during the whole operation of the AGS reactor demonstrate an enrichment in their abundances within the bacterial communities of the granular biomass; remarkably, there was an ample increase of the abundance of *aadA* and *tetA*(A) genes within the resistome of the granule. Hence, before scaling-up

AGS for the *in situ* treatment of hospital effluents, it is mandatory to optimise several operational parameters in order to reduce ARG-enrichment in the bioreactor.

5. The widespread presence of ARGs and their potential hosts in the cryosphere was strongly evidenced by the widespread occurrence of these genetic elements in sludge samples from WWTPs ubicated in cold regions, as well as in undisturbed cold areas, with particular emphasis on their role as a reservoir of antimicrobial resistance under these extreme conditions, reinforcing the global concern about the spread of antimicrobial resistance, even in pristine ecosystems.

7

7-REFERENCES

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