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Wastewater reuse for agricultural irrigation: A comprehensive approach to the mitigation of the antibiotic resistance hazard

Micaela de Almeida Carrapato Oliveira

Dissertation presented to obtain the **Ph.D degree in**

Molecular Biosciences

Oeiras, March, 2023

INSTITUTO DE TECNOLOGIA QUÍMICA E BIOLÓGICA ANTÓNIO XAVIER | UNIVERSIDADE NOVA DE LISBOA

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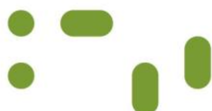

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If you don't like bacteria,

you're on the wrong planet.

Stewart Brand

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ABSTRACT

Water scarcity is considered a serious worldwide problem and an important threat to the development of human societies. The agricultural sector alone accounts for the consumption of about 70 percent of the available freshwater on Earth, being the share of water withdrawal by agriculture still superior to 90 percent in multiple countries. In this context, wastewater reuse for agricultural irrigation comes up as a valuable and sustainable alternative. However, the safety of this practice is still a matter of great concern, since the conventionally applied wastewater treatments are inefficient in the removal of different contaminants of emerging concern, such as antibiotic resistant bacteria and genes, from the treated effluents. Therefore, this thesis performed an assessment on the presence, persistence and characterization of last-line antibiotic resistant bacteria and corresponding resistance genes, from the produced wastewater influents to the reused streams, in two Portuguese full-scale wastewater treatment plants, followed by the application of an additional nanofiltration treatment step to test the removal efficiencies of these antibiotic-related pollutants for the subsequent production of high-quality effluents that could be more safely reused.

First, the population dynamics of the two full-scale wastewater treatment plants was characterized along different sampling points, including the reused effluents, in DNA and extracellular DNA samples. The analysis was performed by high throughput sequencing targeting the 16S rRNA V4 gene region and by three in-house *TaqMan* multiplex qPCR assays that detect and quantify the most clinically relevant and globally distributed carbapenem – $\textit{bla}_{\text{KPC}}$, $\textit{bla}_{\text{OXA-48}}$, $\textit{bla}_{\text{NDM}}$, $\textit{bla}_{\text{IMP}}$, $\textit{bla}_{\text{VIM}}$ – and (fluoro)quinolone – *qnr*A, *qnr*B, *qnr*S – resistance genes. The obtained results identify the biological treatment as the crucial step on tailoring the wastewater bacterial community, which is thereafter maintained in both discharged and reused effluents. Also, the presence of high concentrations of *bla* and *qnr* genes was not only detected in the wastewater influents and discharged effluents, but also in the reused effluents, which therefore represent another gateway for antibiotic resistant bacteria and corresponding genes

into the environment and directly to the human and animal populations. Moreover, it was described, for the first time, the role of the extracellular DNA in the dissemination of carbapenem and (fluoro)quinolone resistance and the impact of the wastewater treatment process on this DNA fraction.

Following the first study on the occurrence and persistence of the carbapenem resistome, two distinct groups of carbapenem resistant bacteria – the potentially environmental and the potentially pathogenic – were isolated from the wastewater influent and discharged effluent of a full-scale wastewater treatment plant and characterized genotypically, through whole genome sequencing, and phenotypically, by antibiotic susceptibility testing. Among the potentially environmental isolates, there was no detection of any acquired antibiotic resistance genes, which supports the idea that their resistance mechanisms are mainly intrinsic. On the contrary, the potentially pathogenic isolates presented a broad diversity of acquired antibiotic resistance genes towards different antibiotic classes, especially β-lactams, aminoglycosides and (fluoro)quinolones. All these bacteria showed multiple β-lactamase-encoding genes, some of which with carbapenemase activity. The antibiotic susceptibility testing assays performed on these isolates also revealed that all had a multiresistance phenotype, indicating that the acquired resistance is their major antibiotic resistance mechanism. Therefore, the two bacterial groups of carbapenem resistant bacteria were proven to have distinct resistance mechanisms, which suggests that the antibiotic resistance in the environment can be a more complex problematic than what is generally assumed.

This thesis also assessed the potential for a commercial Desal 5 DK nanofiltration membrane to be used as a tertiary treatment step in the wastewater treatment plants for a more effective elimination of the antibiotic resistance bacteria and genes from the produced effluents. The study was performed on laboratory scale using a stainless steel cross-flow cell, being the detection and quantification of the total – live and dead – bacteria and of the target carbapenem and (fluoro)quinolone resistance genes performed before and after the applied treatment by flow cytometry and *TaqMan*

multiplex qPCR assays, respectively. High concentrations of total bacteria and of the target carbapenem and (fluoro)quinolone resistance genes (*bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *qnr*A, *qnr*B and *qnr*S) were detected not only in the discharged, but also in the reused effluent samples, suggesting that their reuse may not be entirely safe. Nevertheless, the applied nanofiltration treatment achieved removal rates superior to 98 percent for the total bacteria and 99.99 percent for all the target resistance genes present in both DNA and extracellular DNA factions, with no significant differences for these microbiological parameters between nanofiltered and tap water samples.

Altogether, the results obtained in this thesis not only undoubtedly showed the importance of the wastewater treatment plants and of the environmental sector on the antibiotic resistance spreading cycle, but also demonstrated that, for wastewater reuse to become a safe and reliable practice, targeted treatments towards antibiotic resistant bacteria and genes must be developed and implemented in the wastewater treatment plants. Therefore, and despite the need for additional studies at pilot scale to fully optimize the entire process, nanofiltration membranes seem to have a great potential to be used as a tertiary treatment step, allowing the production of a high-quality reclaimed water that can be more safely reused for agricultural irrigation.

RESUMO

A escassez de água é considerada um grave problema mundial e uma importante ameaça ao desenvolvimento das sociedades. O sector agrícola é responsável pelo consumo de cerca de 70 por cento da água doce disponível no planeta Terra, sendo a percentagem de água utilizada pela agricultura superior a 90 por cento em múltiplos países. Neste contexto, a reutilização de água residual para irrigação agrícola surge como sendo uma alternativa valiosa e sustentável. No entanto, a segurança desta prática ainda é motivo de grande preocupação mundial, uma vez que os tratamentos convencionalmente utilizados para o tratamento de águas residuais são ineficientes na remoção de diferentes contaminantes emergentes, tais como bactérias e genes de resistência a antibióticos, dos efluentes tratados. Desta forma, esta tese realizou uma avaliação da presença, persistência e caracterização de bactérias resistentes a antibióticos de última linha e dos respectivos genes de resistência, desde os influentes até aos efluentes para reutilização, em duas estações de tratamento de águas residuais portuguesas. Posteriormente, foi ainda aplicada uma etapa adicional de tratamento por nanofiltração, de forma a testar a eficiência da remoção destes micropoluentes para a subsequente produção de efluentes de elevada qualidade, que possam ser reutilizados com maior segurança.

Em primeiro lugar, a dinâmica populacional das duas estações de tratamento de águas residuais foi caracterizada ao longo de diferentes pontos de amostragem, incluindo os efluentes para reutilização, em amostras de ADN e ADN extracelular. A análise foi realizada por sequenciação de alto rendimento da região V4 do gene 16S rRNA e por três ensaios *TaqMan multiplex qPCR* desenvolvidos internamente para detectar e quantificar os genes de resistência aos carbapenemes – bla_{KPC}, $bla_{\mathrm{OXA\text{-}48}}$, bla_{NDM}, bla_{IMP}, bla_{VIM} – e às (fluoro)quinolonas – *qnr*A, *qnr*B, *qnr*S – clinicamente mais relevantes e com maior distribuição global. Os resultados obtidos identificam o tratamento biológico como sendo a etapa crucial na determinação da comunidade bacteriana constituinte das águas residuais, que se mantém praticamente inalterada

nos efluentes para descarga e para reutilização. Além disso, a presença de elevadas concentrações de genes *bla* e *qnr* foi detectada não só nos influentes e nos efluentes para descarga, como também nos efluentes para reutilização, que representam assim mais uma porta de entrada de bactérias e genes de resistência a antibióticos para o meio ambiente e directamente para as populações humanas e animais. Neste estudo, foi também descrito, pela primeira vez, o papel do ADN extracelular na disseminação da resistência aos carbapenemes e às (fluoro)quinolonas e o impacto do processo de tratamento de águas residuais nesta fracção de ADN.

Após o primeiro estudo acerca da ocorrência e persistência do resistoma aos carbapenemes, dois grupos distintos de bactérias resistentes aos carbapenemes – as potencialmente ambientais e as potencialmente patogénicas – foram isoladas do influente e do efluente para descarga de uma estação de tratamento de águas residuais e caracterizados genotipicamente, através de sequenciação completa do genoma, e fenotipicamente, através de testes de susceptibilidade a antibióticos. De entre os isolados potencialmente ambientais, não foi detectado nenhum gene adquirido de resistência a antibióticos, o que corrobora a ideia de que os seus mecanismos de resistência são maioritariamente intrínsecos. No entanto, os isolados potencialmente patogénicos apresentaram uma ampla diversidade de genes de resistência a diferentes classes de antibióticos, especialmente aos antibióticos β-lactâmicos, aminoglicosídeos e (fluoro)quinolonas. Todas estas bactérias apresentaram múltiplos genes codificantes para β-lactamases, algumas das quais também com actividade carbapenemase. Os ensaios de susceptibilidade realizados nestes isolados revelaram ainda que todos têm um fenótipo de multirresistência, o que indica que a resistência adquirida é o seu principal mecanismo de resistência aos antibióticos. Deste modo, os dois grupos de bactérias resistentes aos carbapenemes demonstraram ter mecanismos de resistência distintos, o que sugere que a resistência aos antibióticos no ambiente possa ser uma problemática mais complexa do que o que geralmente se assume.

Nesta tese, foi também avaliado o potencial de uma membrana comercial de nanofiltração Desal 5 DK para ser utilizada como etapa de tratamento terciário nas estações de tratamento de águas residuais para uma eliminação mais eficaz de bactérias e genes de resistência a antibióticos dos efluentes produzidos. O estudo foi realizado à escala laboratorial usando uma célula de fluxo cruzado de aço inoxidável, sendo a detecção e quantificação das bactérias totais – vivas e mortas – e de genes de resistência aos carbapenemes e às (fluoro)quinolonas realizada antes e depois do tratamento por citometria de fluxo e através de ensaios de *TaqMan multiplex qPCR*, respectivamente. Foram detectadas elevadas concentrações de bactérias totais e de genes de resistência aos carbapenemes e às (fluoro)quinolonas (bla_{KPC}, $bla_{OXA\text{-}48}$, bla_{NDM}, bla_{IMP}, bla_{VIM}, *qnr*A, *qnr*B and *qnr*S) não só nas amostras de efluente para descarga, como também nas amostras de efluente para reutilização, o que sugere que o seu uso possa não ser completamente seguro. No entanto, o tratamento de nanofiltração atingiu taxas de remoção superiores a 98 por cento para as bactérias totais e 99,99 por cento para todos os genes de resistência presentes nas fracções de ADN e ADN extracelular, sem que se verificassem diferenças significativas para estes parâmetros microbiológicos entre amostras de água nanofiltrada e amostras de água da torneira.

No seu conjunto, os resultados obtidos nesta tese não só mostram, sem margem para dúvidas, a importância das estações de tratamento de águas residuais e do sector ambiental no ciclo de propagação da resistência aos antibióticos, como também demonstram que, para que a reutilização de águas residuais se torne uma prática segura e fiável, tratamentos dirigidos à eliminação de bactérias e genes de resistência aos antibióticos devem ser desenvolvidos e implementados nas estações de tratamento de águas residuais. Assim, e apesar da necessidade de estudos adicionais à escala piloto para optimizar todo o processo, as membranas de nanofiltração parecem ter um grande potencial para serem usadas como tratamento terciário, uma vez que produzem uma água de elevada qualidade que pode ser reutilizada com mais segurança para irrigação agrícola.

THESIS PUBLICATIONS

Publications in peer-reviewed journals

Oliveira, M., Leonardo, I.C., Silva, A.F., Crespo, J.G., Nunes, M., Crespo, M.T.B., 2022. Nanofiltration as an Efficient Tertiary Wastewater Treatment: Elimination of Total Bacteria and Antibiotic Resistance Genes from the Discharged Effluent of a Full-Scale Wastewater Treatment Plant. *Antibiotics* 11, 630.
doi: 10.3390/antibiotics11050630

Oliveira, M., Leonardo, I.C., Nunes, M., Silva, A.F., Barreto Crespo, M.T., 2021. Environmental and Pathogenic Carbapenem Resistant Bacteria Isolated from a Wastewater Treatment Plant Harbour Distinct Antibiotic Resistance Mechanisms. *Antibiotics* 10, 1118.
doi: 10.3390/antibiotics10091118

Oliveira, M., Nunes, M., Barreto Crespo, M.T., Silva, A.F., 2020. The environmental contribution to the dissemination of carbapenem and (fluoro)quinolone resistance genes by discharged and reused wastewater effluents: The role of cellular and extracellular DNA. *Water Res.* 182, 116011.
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Other publications in peer-reviewed journals

Cristóvão, M.B., Tela, S., Silva, A.F., **Oliveira, M.**, Bento-Silva, A., Bronze, M.R., Crespo, M.T.B., Crespo, J.G., Nunes, M., Pereira, V.J., 2021. Occurrence of antibiotics, antibiotic resistance genes and viral genomes in wastewater effluents and their treatment by a pilot scale nanofiltration unit. *Membranes* 11, 1-16.
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Poster communications

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wastewater treatment process. 8th Congress of European Microbiologists (FEMS 2019), Glasgow, Scotland, 7-11 July 2019.

Micaela Oliveira, Mónica Nunes, Maria Teresa Barreto Crespo, Ana Filipa Silva, 2019. Detection and quantification of antibiotic resistance genes along the wastewater treatment process by *TaqMan* multiplex qPCR. 15th Symposium on Bacterial Genetics and Ecology (BAGECO 15), Lisbon, Portugal, 26-30 May 2019.

LIST OF ABBREVIATIONS

A. baumannii	*Acinetobacter baumannii*
A. caviae	*Aeromonas caviae*
A. oleivorans	*Acinetobacter oleivorans*
A. pittii	*Acinetobacter pittii*
A. salmonicida	*Aeromonas salmonicida*
A. veronii	*Aeromonas veronii*
AB	Antibiotic
AMP10	Ampicillin 10 µg
ANOVA	Analysis of variance test
BAF	Biological aerated filters
BNR AS	Biological nutrient removal by activated sludge
BOD_5	Biological oxygen demand (5 days)
BP	Base pairs
C. amalonaticus	*Citrobacter amalonaticus*
C. freundii	*Citrobacter freundii*
C. portucalensis	*Citrobacter portucalensis*
C. rhizoryzae	*Citrobacter rhizoryzae*
C30	Chloramphenicol 30 µg
CFU	Colony-forming unit
CIP5	Ciprofloxacin 5 µg
CN10	Gentamicin 10 µg
COD	Chemical oxygen demand
CTX5	Cefotaxime 5 µg
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
eDNA	Extracellular deoxyribonucleic acid
EARS-Net	European Antimicrobial Resistance Surveillance Network
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization of the United Nations
I	Susceptible, increased exposure
IMP10	Imipenem 10 µg
K. pasteurii	*Klebsiella pasteurii*
K. pneumoniae	*Klebsiella pneumoniae*
MEM10	Meropenem 10 µg
NCBI	National Centre for Biotechnology

P. entomophila	*Pseudomonas entomophila*
P. fluorescens	*Pseudomonas fluorescens*
P. fragi	*Pseudomonas fragi*
P. lundensis	*Pseudomonas lundensis*
P. migulae	*Pseudomonas migulae*
P. monteilii	*Pseudomonas monteilii*
P. psychrophila	*Pseudomonas psychrophila*
P. putida	*Pseudomonas putida*
P. syringae	*Pseudomonas syringae*
PBP	Penicillin-binding protein
PCA	Principal component analysis
PCR	Polymerase chain reaction
PE	Population equivalents
PES	Polyethersulfone
PI	Propidium iodide
PT	Pressure transducer
qPCR	Quantitative real-time polymerase chain reaction
R	Resistant
RNA	Ribonucleic acid
S	Susceptible, standard dosing regimen
SX25	Trimethoprim + sulphamethoxazole 25 µg
TE	Tris-EDTA
TE30	Tetracycline 30 µg
TSB	Tryptic soy broth
TSS	Total suspended solids
W5	Trimethoprim 5 µg
WHO	World Health Organization
WWTP	Wastewater treatment plant

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CHAPTER 1

Introduction: State-of-the-art and Thesis motivation

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1.1 THE PROBLEM OF WATER SCARCITY

Water scarcity is considered a serious worldwide problem and a major threat to the development of human societies (Mancosu et al., 2015; Mekonnen and Hoekstra, 2016). This situation is influenced not only by climate change, but also by the global increase in the water demand observed over the last decades, mainly due to the population growth and consequent rising needs for agricultural products (for food and non-food uses), continuous shifts in consumption patterns (for more meat and sugar-based products), urbanization and water pollution (de Fraiture and Wichelns, 2010; Ercin and Hoekstra, 2014; Mekonnen and Hoekstra, 2016; Ungureanu et al., 2020). For instance, water stress, which is defined as the ratio between the total freshwater withdrawn by all major sectors and the total renewable freshwater resources (after taking into account the environmental water requirements), is already severely affecting most Western / Central Asian and Northern African countries and starting to affect Europe and Northern America (Figure 1.1) (FAO, 2021).

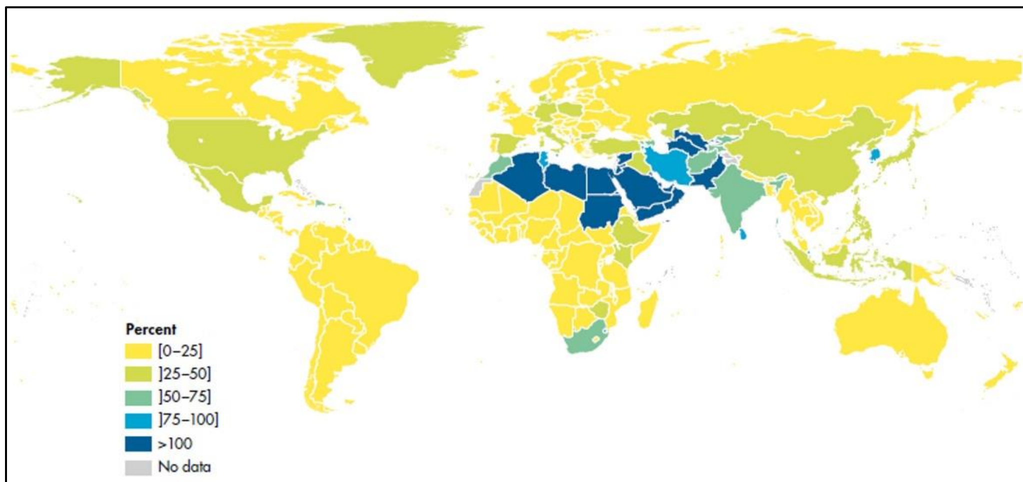

Figure 1.1 | Water stress levels worldwide (2018). Adapted from (FAO, 2021).

The agricultural sector accounts for the consumption of about 70 percent of the available freshwater on Earth, being the share of water withdrawal by agriculture still

superior to 90 percent in 31 countries (FAO, 2021, 2011). Furthermore, by 2050, the world population is expected to have grown to more than 9 billion people, which will require an increase in food production of approximately 70 percent (100 percent in developing countries) regarding the 2009 levels (FAO, 2011; Ungureanu et al., 2020). In this scenario, attention is turning to the pursuit of complementary water sources for agricultural irrigation, being wastewater reuse a valuable and sustainable alternative to face the challenges ahead.

1.2 WASTEWATER REUSE AS AN ALTERNATIVE FOR AGRICULTURAL IRRIGATION

1.2.1 Benefits

Great volumes of wastewater are daily generated and disposed by households, industries and agriculture (Ungureanu et al., 2020). The global wastewater discharge is estimated at, approximately, 400 billion m^3 / year, resulting in a great pressure on the environment and in the pollution of around 5,500 m^3 of water / year (Zhang and Shen, 2017). Being already well known that the discharge of these wastewater effluents can cause severe degradation of rivers, lakes and coastal marine environments, their reuse after the application of additional treatment steps can have a considerable effect on reducing the harmful impacts of their discharge into the receiving environments (Toze, 2006). Moreover, and since wastewater effluents are being constantly produced, their reuse for agricultural irrigation purposes can significantly reduce the massive share of freshwater withdrawal by this sector, representing a valuable approach to mitigate the problems posed by water scarcity (Lavrnić et al., 2017; Toze, 2006). In fact, wastewater effluents are usually rich in both organic and inorganic nutrients, such as nitrogen and phosphorus, with potential to be used as an inexpensive fertilizer for the increase of crop productivity, therefore saving costs, energy and minimizing carbon emissions to the environment (Hanjra et al., 2012; Toze, 2006). Accordingly, and due to its multiple benefits, this practice has been observed an increasing acceptance over the years in

many countries, which are already reusing both treated and untreated wastewater effluents for agricultural irrigation (Figure 1.2) (Jaramillo and Restrepo, 2017).

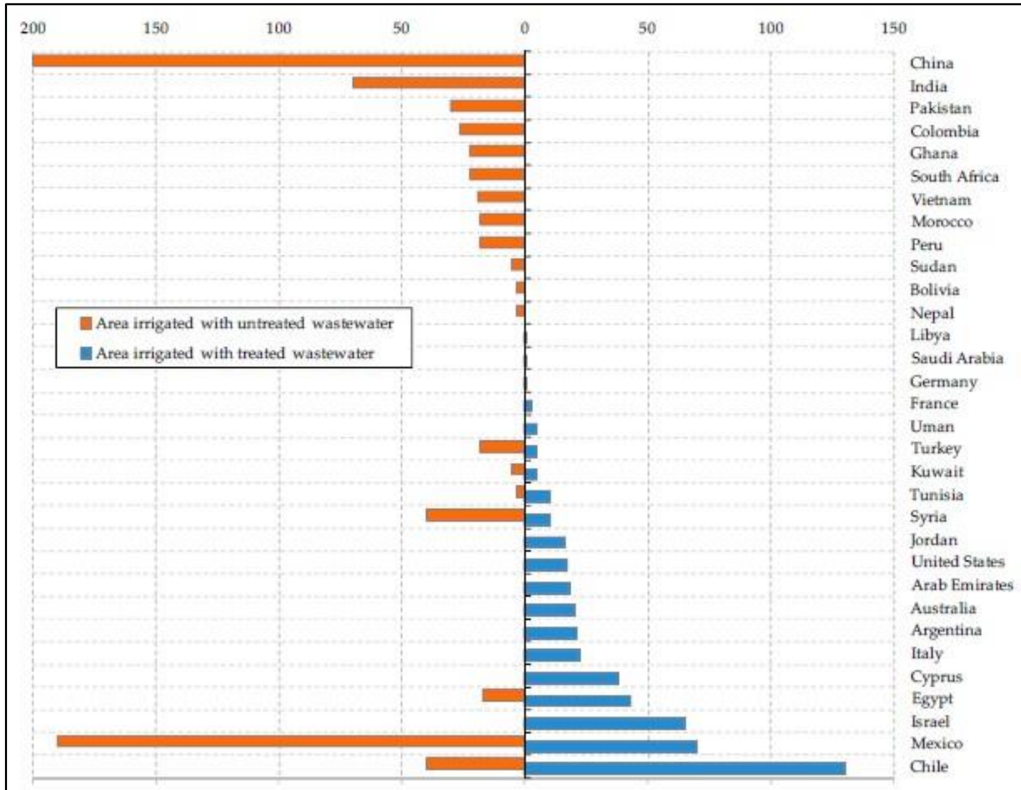

Figure 1.2 | Area (thousands of ha) irrigated with untreated (orange) and treated (blue) wastewater effluents in countries that already reuse wastewater for agricultural irrigation. Adapted from (Jaramillo and Restrepo, 2017).

1.2.2 Current roadblocks

Despite all benefits resulting from being a valuable and sustainable practice, the safety of wastewater reuse for agricultural irrigation is still a matter of great concern worldwide. If not properly treated, wastewater reuse can cause severe problems to soils (e.g., salinization, toxicity due to sodium, chloride and boron ions, structural degradation, reduced aeration / pore-clogging due to suspended solids, reduced soil

hydraulic conductivity), groundwater reservoirs (e.g., leaching of excessive nitrates), crops (e.g., high microbial load, nutrient and heavy metal accumulation) and endanger the health of farm workers and crop consumers (humans and animals) (Hanjra et al., 2012; Jaramillo and Restrepo, 2017; Ungureanu et al., 2020). In fact, when untreated or improperly treated, wastewater effluents can harbour a wide range of pathogens, able to survive in different environments and be transmitted to humans and animals, either by direct contact with the contaminated water or by ingestion of irrigated products, causing potentially fatal bacterial, viral or parasitic infections (Hanjra et al., 2012; Ungureanu et al., 2020; WHO, 2006). This situation is particularly worrying in most developing countries, where outbreaks of cholera, typhoid fever and shigellosis, as well as seropositive responses for *Helicobacter pylori* and increases in non-specific diarrhea cases are frequently reported due to the use of untreated wastewater effluents for agricultural irrigation (Kamizoulis, 2008; Ungureanu et al., 2020). Thus, and despite this being a practice strongly encouraged by the official entities worldwide, only a few countries have already implemented directives on the physicochemical and biological parameters of treated wastewater effluents, to protect not only the human and animal health, but also the environment, upon its use. Meanwhile, most low-income countries continue to lack the resources to properly treat their wastewaters, reusing the untreated wastewater effluents, with all the risks this practice entails.

1.2.3 Regulations and the problem of the emerging contaminants

In May 2020, the European Commission published its new regulation on minimum requirements for water reuse, to create an enabling framework for the Member States who wish or need to practice water reuse (EC, 2020). Table 1.1 shows the defined classes of reclaimed water quality and corresponding permitted agricultural uses and irrigation methods, and Table 1.2 presents the reclaimed water quality requirements for each class.

Table 1.1 | Classes of reclaimed water quality and corresponding permitted agricultural use and irrigation method. Adapted from (EC, 2020).

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

(*) If the same type of irrigated crop falls under multiple categories of Table 1.1, the requirements of the most stringent category shall apply.

(**) Drip irrigation (also called trickle irrigation) is a micro-irrigation system capable of delivering water drops or tiny streams to the plants and involves dripping water onto the soil or directly under its surface at very low rates (2-20 liters / hour) from a system of small-diameter plastic pipes fitted with outlets called emitters or drippers.

(***) In the case of irrigation methods which imitate rain, special attention should be paid to the protection of the health of workers or bystanders. For this purpose, appropriate preventive measures shall be applied.

Table 1.2 | Reclaimed water quality requirements for agricultural irrigation. Adapted from (EC, 2020).

<table>
<tr><th rowspan="2">Reclaimed water quality class</th><th rowspan="2">Indicative technology target</th><th colspan="5">Quality requirements</th></tr>
<tr><th>E. coli (number / mL)</th><th>BOD5 (mg / L)</th><th>TSS (mg / L)</th><th>Turbidity (NTU)</th><th>Other</th></tr>
<tr><td>A</td><td>Secondary treatment, filtration and disinfection</td><td>≤ 10</td><td>≤ 10</td><td>≤ 10</td><td>≤ 5</td><td rowspan="2">Legionella spp.: < 1,000 CFU / L where there is a risk of aerosolisation</td></tr>
<tr><td>B</td><td>Secondary treatment and disinfection</td><td>≤ 100</td><td rowspan="3">In accordance with Directive 91/271/EEC</td><td rowspan="3">In accordance with Directive 91/271/EEC</td><td>-</td></tr>
<tr><td>C</td><td>Secondary treatment and disinfection</td><td>≤ 1,000</td><td>-</td><td rowspan="2">Intestinal nematodes (helminth eggs): ≤ 1 egg / L for irrigation of pastures or forage</td></tr>
<tr><td>D</td><td>Secondary treatment and disinfection</td><td>≤ 10,000</td><td>-</td></tr>
</table>

BOD_5: Biological oxygen demand (5 days)

TSS: Total suspended solids

Despite being a good starting point, this regulation still fails to account for the presence of different contaminants of emerging concern, such as pharmaceutically active compounds, endocrine disruptors, personal care products and heavy metals, in the treated wastewater effluents. Although these substances were not considered contaminants in the past, due to the absence of information on the effects of their accumulation on the soils, water, air and tissues, their concentrations have more recently started to be addressed in multiple water sources (Jaramillo and Restrepo, 2017). Moreover, regarding the microbiological contamination parameters, only the concentrations of coliforms (namely, *E. coli*) and *Legionella* spp. are being considered in the routine analysis, which is insufficient and does not allow the evaluation of the presence of other pathogenic microorganisms in the treated wastewater effluents. In fact, and taking the case of antibiotics, several studies point out for the inefficiency of the conventional wastewater treatments in their removal, as well as of the associated resistant bacteria and corresponding resistance genes, from the treated effluents (for discharge and reuse) (Auguet et al., 2017; Cacace et al., 2019; Lamba and Ahammad, 2017; Mao et al., 2015; Mathys et al., 2019; Munir et al., 2011; Naquin et al., 2017; Neudorf et al., 2017; Pärnänen et al., 2019; Rafraf et al., 2016; Yang et al., 2016; Zieliński et al., 2021). This not only further highlights the importance of an appropriate treatment of the wastewater effluents, but also suggests that enhanced wastewater treatments should be developed and applied in the wastewater treatment plants to produce effluents that can be more safely discharged and / or reused.

1.3 WASTEWATER TREATMENT PLANTS AS HOTSPOTS OF ANTIBIOTIC RESISTANCE DISSEMINATION INTO THE ENVIRONMENT

1.3.1 Antibiotic resistance in wastewater treatment plants

Over the last decades, antibiotics have been extensively used in both human and veterinary medicine, as well as in areas such as agriculture, livestock and aquaculture.

As a result of this intensive use, and since most antibiotics are poorly absorbed and not entirely metabolized by the human and animal bodies, considerable fractions of these pharmaceuticals are excreted into sewage in their unmetabolized original forms or as active and inactive metabolites, ending up in wastewater treatment plants (Christou et al., 2017; Grossberger et al., 2014; Jelic et al., 2011). In this context, the bacterial wastewater communities are continuously exposed to sub-inhibitory concentrations of antibiotics, which promotes a selective environment for resistant microorganisms and the spread of antibiotic resistances (Davies et al., 2006; Michael et al., 2013; Rizzo et al., 2013). Moreover, since the conventional wastewater treatments were originally mainly designed for the removal of carbon, nitrogen and phosphorus, and not for the elimination of contaminants of emerging concern, such as antibiotic resistant bacteria and corresponding resistance genes, wastewater treatment plants are considered major anthropogenic reservoirs and sources for the dissemination of these pollutants into the environment (Alexander et al., 2020; Guo et al., 2017; Michael et al., 2013; Uluseker et al., 2021). The presence of antibiotic resistant bacteria and genes towards the most commonly used classes of antibiotics, namely β-lactams, aminoglycosides, (fluoro)quinolones, macrolides, sulphonamides, tetracyclines and trimethoprim, have already been extensively reported along all the main steps of different wastewater treatment processes worldwide (Auguet et al., 2017; Cacace et al., 2019; Mao et al., 2015; Munir et al., 2011; Neudorf et al., 2017; Rafraf et al., 2016; Zieliński et al., 2021). However, the even more concerning situation of the resistance towards multiple last-line antibiotics, such as carbapenems, has not yet been fully explored, being required further studies to understand the occurrence of these resistant bacteria and genes in distinct geographic locations and their fate along the most critical steps of different wastewater treatment processes.

1.3.2 Antibiotic resistance towards last-line antibiotics – Carbapenems

Carbapenems are one of the most important classes of antibiotics used to treat the most severe multiresistant infections caused by both Gram-positive and Gram-negative bacteria (Kattan et al., 2008; Papp-Wallace et al., 2011). Nevertheless, their efficiency is currently threatened by the global emergence and spread of different carbapenem resistant bacteria, which can express enzyme-mediated and / or non-enzyme mediated resistance mechanisms towards these antibiotics (Nordmann et al., 2012; Nordmann and Poirel, 2019). The enzyme-mediated resistance mechanisms are encoded by *bla* genes and involve the hydrolysis of carbapenems by carbapenemases, a particular group of β-lactamases that also hydrolyze other β-lactam antibiotics, such as penicillins, cephalosporins and monobactams (Nordmann et al., 2012; Nordmann and Poirel, 2019; Papp-Wallace et al., 2011; Poirel et al., 2007; Walsh, 2010). In terms of clinical relevance and global distribution, the most important carbapenemases are: (1) Class A serine-β-lactamases encoded by bla_{KPC}-type genes; (2) Class B metallo-β-lactamases, encoded by bla_{NDM}, bla_{IMP} and bla_{VIM}-type genes; (3) Class D serine-β-lactamases, encoded by bla_{OXA-48}-type genes (Nordmann et al., 2012; Pfeifer et al., 2010). These enzyme-mediated resistance mechanisms pose the greatest threat, since not only carbapenemases are able to inactivate the majority of β-lactam antibiotics, but their encoding-genes are often found in plasmids, transposons or other mobile genetic elements, which promotes their horizontal transfer between bacteria. In fact, it is already known that the plasmid transfer and acquisition is the main driver of the rapid increase and spread of the carbapenem resistance that has been observed over the last decades (Nordmann et al., 2012; Nordmann and Poirel, 2019; Pfeifer et al., 2010; Potter et al., 2016; Schultsz and Geerlings, 2012; Yang et al., 2021). However, bacteria can also be resistant to carbapenems due to: (1) Mutations causing loss of expression of porin-encoding genes, production of modified porins and / or shifts in the types of porins found in the outer membrane; (2) The overexpression of genes encoding for efflux pumps; (3) Mutations that modify the production levels and / or the binding

affinities of the penicillin-binding proteins, preventing their binding to the antibiotics, therefore allowing the synthesis of the peptidoglycan layer of the bacterial cell wall (Nordmann et al., 2012; Nordmann and Poirel, 2019). These non-enzyme mediated resistance mechanisms, which are also known as intrinsic resistance mechanisms, can occur alone or together with the production of extended-spectrum β-lactamases, cephalosporinases and / or carbapenemases, generating well-known carbapenem resistance phenotypes (Nordmann et al., 2012; Nordmann and Poirel, 2019). Examples of enzyme-mediated and non-enzyme mediated carbapenem resistance mechanisms are represented in Figure 1.3.

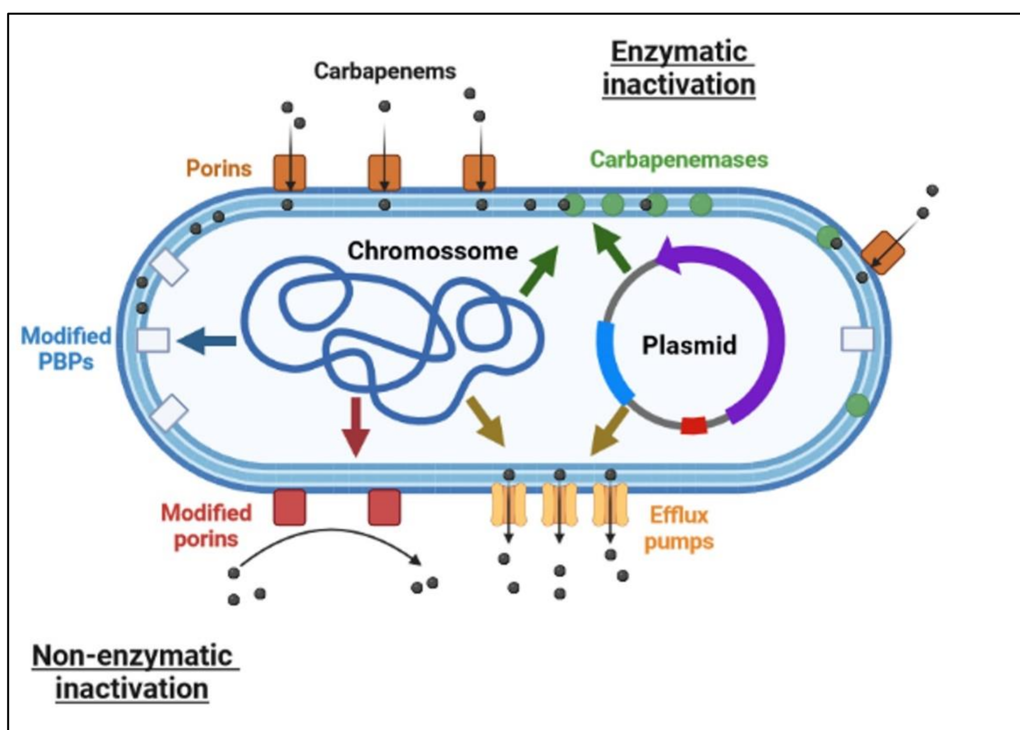

Figure 1.3 | Scheme of the enzyme-mediated (carbapenemases) and non-enzyme-mediated (modified porins, efflux pumps and modified penicillin-binding proteins (PBPs)) carbapenem resistance mechanisms. Adapted from (Nordmann et al., 2012).

Multiple studies, mostly carried out in urban wastewater treatment plants based on activated sludge processes, with different configurations and, in a few cases, with disinfection and / or tertiary treatment stages, have already reported the detection of carbapenem resistant bacteria and corresponding resistance genes in the wastewater influents and treated effluents of different European (Auguet et al., 2017; Cacace et al., 2019; Pärnänen et al., 2019), Asian (Lamba and Ahammad, 2017; Yang et al., 2016) and Northern American (Mathys et al., 2019; Naquin et al., 2017) countries. However, there are still important gaps in the current knowledge that have to be overcome to better understand the role of carbapenem resistant bacteria and genes in the contamination of the treated wastewater effluents and to clarify whether or not these pollutants represent an additional hazard to consider when thinking about wastewater reuse for agricultural irrigation purposes: (1) The search for all main carbapenem resistance genes, to provide a more accurate picture regarding the carbapenem resistome present in the wastewater treatment plants; (2) The look for the possible co-occurrence of resistance genes to carbapenems and other antibiotics, such as (fluoro)quinolones, since resistance to these two antibiotic classes is often simultaneously transferred between bacteria; (3) The analysis of treated effluents, not only for discharge, but also for reuse, since they can represent an additional gateway for antibiotic resistance bacteria and genes into the environment and directly to the human populations; (4) The focus not only on the cellular DNA, but also on the free / extracellular DNA, which can harbour an important fraction of antibiotic resistance genes that are able to be assimilated by non-resistant bacteria by natural transformation; (5) The isolation of the different populations of carbapenem resistant bacteria that exist in the wastewater environments and the subsequent assessment and characterization of their acquired resistance genes and intrinsic resistance mechanisms towards carbapenems and other antibiotics. This thesis addresses these topics through a deep insight on the occurrence / persistence of the carbapenem resistome and characterization of the corresponding resistant bacteria along two Portuguese full-scale wastewater treatment plants.

1.4 WASTEWATER TREATMENTS

1.4.1 Conventional wastewater treatments

Until recent years, the objective of wastewater treatment plants was mainly the reduction of the content of suspended solids, oxygen-demanding materials, dissolved inorganic compounds and bacteria from the treated wastewater effluents (Sonune and Ghate, 2004). To achieve this goal, wastewater treatment systems using activated sludge processes have been extensively employed worldwide, since they can produce effluents able to meet the currently required quality standards (for both discharge in the environment and reuse) at acceptable operating and maintenance costs (Jelic et al., 2012). A scheme of a conventional wastewater treatment process is represented in Figure 1.4.

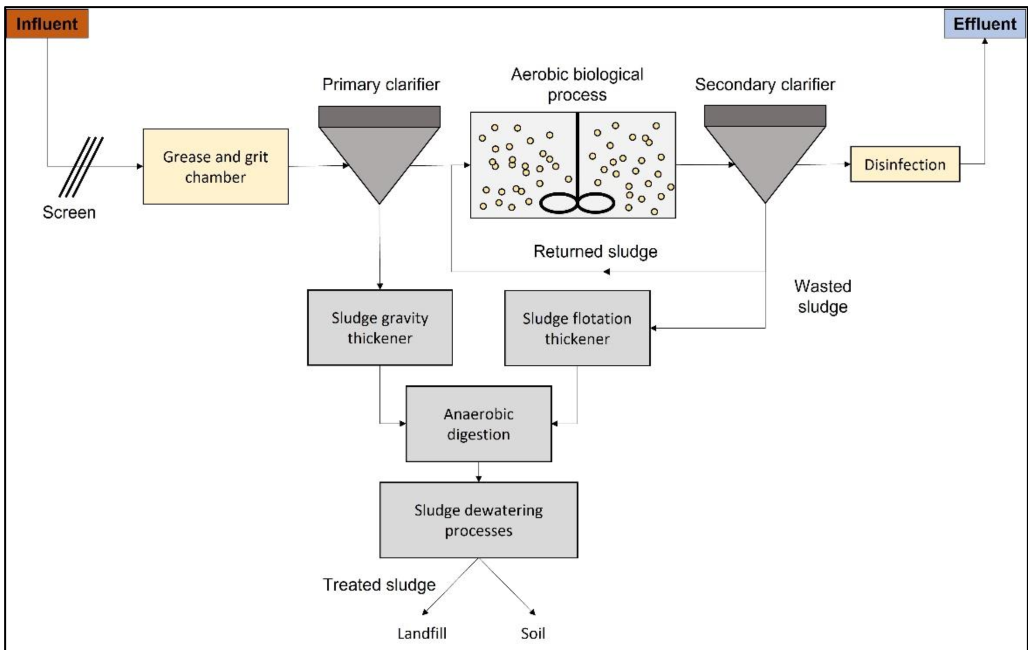

Figure 1.4 | Scheme of a conventional wastewater treatment process, from the wastewater influent to the treated wastewater effluent. Adapted from (Jelic et al., 2012).

The first step consists in a preliminary treatment with a bar screen to eliminate the coarse solids and other large materials that are usually found in the wastewater influents (e.g., pieces of wood, cloth, paper, plastic, garbage, fecal matter, etc.). The wastewater influent then passes through a primary treatment unit, composed by a chamber and a settling reactor (primary clarifier) designed to eliminate most organic and inorganic solids by the physical processes of sedimentation and flotation. At this stage, around 25-50 percent of the incoming biochemical oxygen demand, 50-70 percent of the total suspended solids and 65 percent of the oil, grease and grit are removed. Part of the organic nitrogen, phosphorus and heavy metals associated with solids are also removed during this primary sedimentation, although the colloidal and dissolved constituents are not affected. The primary-treated effluent then enters the secondary treatment, also known as biological treatment, where the microorganisms responsible for the treatment, along with the biodegradable and nonbiodegradable suspended, colloidal and soluble organic and inorganic matter – the mixed liquor – are maintained in a liquid suspension by appropriated mixing methods, leading to the adsorption, flocculation and oxidation of the organic matter. After that, the mixed liquor is transferred to a settling reactor (secondary clarifier) to allow the separation of the suspended solids (now in the form of floc particles) from the already treated wastewater by the action of gravity. In the end, part of the settled solids is returned to the biological reactor (return activated sludge) to maintain a concentrated biomass for the wastewater treatment and the remaining is wasted, since microorganisms are continuously multiplying and a specific biomass concentration should be strictly maintained in the system. The waste sludge is therefore discharged to the primary sedimentation tanks or other facilities for co-thickening, to increase the solid content of sludge by removing a portion of the liquid fraction. Through processes of digestion, dewatering, drying and combustion, the water and organic content are considerably reduced, being the processed solids suitable for reuse or final disposal (Crini and Lichtfouse, 2019; Jelic et al., 2012; Sonune and Ghate, 2004).

The conventional primary and secondary wastewater treatments remove most of the biological oxygen demand and total suspended solids present in the wastewaters (Sonune and Ghate, 2004). However, to produce effluents that can be more safely reused, for example, in agricultural irrigation, further removal efficiencies should be achieved and the elimination of multiple contaminants of emerging concern, such as antibiotic resistant bacteria and genes, have to be addressed. Therefore, additional advanced treatment steps – tertiary treatments – should be developed and added to the already conventionally applied treatments in the wastewater treatment plants. Among the already existing technologies, membrane separation processes, ozonation, adsorption and advanced oxidation processes are considered the most mature and ready to be implemented, having been successfully tested for the removal of several pharmaceutically active compounds from the wastewaters (Garcia-Ivars et al., 2017; González et al., 2015; Sun et al., 2022; Xu et al., 2020). Briefly, membrane separation processes involve the passage of the treated wastewater through a membrane for the elimination of particulate material, pathogens, organic matter, nutrients and dissolved substances not removed by the previous treatments; ozonation consists in a chemical treatment involving the infusion of ozone in the treated wastewater; adsorption relies on the accumulation of substances present in the treated wastewater (adsorbates) on a suitable interface (adsorbent), being considered a mass transfer operation, from a fluid to a solid phase; advanced oxidation processes are based on an initial generation of hydroxyl radicals, which then become the main oxidizing agent of the system, able to remove a considerable number of contaminants. Currently, membrane separation processes, such as ultrafiltration, nanofiltration and reverse osmosis, are considered the most powerful technologies for the removal of different microcontaminants from wastewaters, being nanofiltration one of the most cost-efficient methods to perform enhanced wastewater treatments, since it represents a good compromise between the required water quality and the energy expenditure necessary for its production (Garcia-Ivars et al., 2017; González et al., 2015; Mohammad et al., 2015; Xu et al., 2020).

1.4.2 Advanced wastewater treatments – Nanofiltration

Nanofiltration membranes present separation properties between those observed for ultrafiltration and reverse osmosis membranes, with a pore size in the order of 1 nm, which corresponds to a molecular weight cut-off between 100 and 5,000 Da (Figure 1.5). They also exhibit a moderate level of charge due to the dissociation of surface functional groups or the adsorption of charged solutes, operate with no phase change and have high rejections of multivalent inorganic salts and small organic molecules at relatively low pressures. All these features make their separation process extremely competitive in terms of selectivity and cost-benefit when compared to other separation methods (Foureaux et al., 2019; Mohammad et al., 2015; Oatley-Radcliffe et al., 2017).

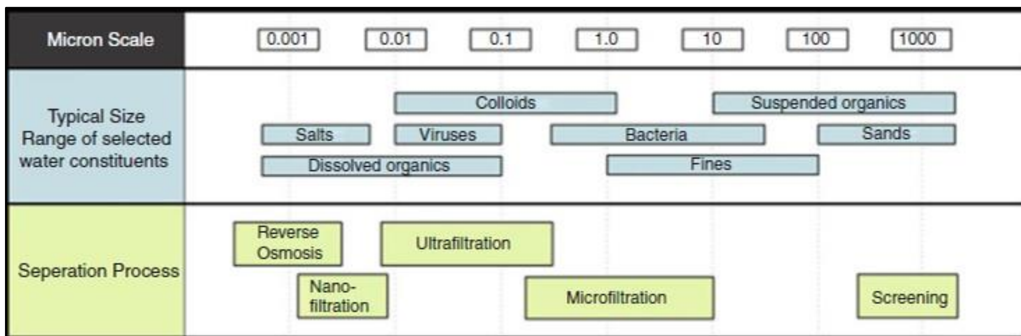

Figure 1.5 | Size range of the wastewater constituents and operation range of the different membrane separation processes. Adapted from (González et al., 2015).

Since nanofiltration is expected to be an effective technique for the removal of multiple contaminants of emerging concern from the wastewaters, it can be used to produce high-quality effluents in a more sustainable way than reverse osmosis, due to its higher permeate flux and ability to work at lower pressures, which contributes for a decrease in the energy consumption (Couto et al., 2018; Foureaux et al., 2019; Oatley-Radcliffe et al., 2017). In fact, recent studies using nanofiltration membranes as a tertiary wastewater treatment technique have already been showing promising results regarding the removal efficiencies of important emerging contaminants, such as

pharmaceutically active compounds, endocrine disruptors, personal care products and heavy metals (Cristóvão et al., 2019; Cuhorka et al., 2020; Foureaux et al., 2019; Garcia-Ivars et al., 2017; Xu et al., 2020). However, only a few have already started to address the great threat of antibiotic resistance and, in particular, of the resistance towards last-line antibiotics, by focusing on the removal efficiencies of the antibiotic resistant bacteria and corresponding resistance genes from the treated wastewater effluents (Cristóvão et al., 2021; Slipko et al., 2019). This thesis addresses this challenge and its potential drawbacks by providing a better understanding on whether or not the application of this final nanofiltration treatment step can be considered an alternative to produce high-quality effluents, free of antibiotic-related pollutants, that could be more safely reused in agricultural irrigation.

1.5 THESIS MOTIVATION

Considering the state-of-the-art presented, it is possible to conclude that, despite being a valuable and sustainable alternative to overcome the global problem of water scarcity, wastewater reuse for agricultural irrigation purposes is a practice that still raises important public health issues regarding its safety, even if it complies with the current legislation on water reuse, which fails to account for the presence of multiple contaminants of emerging concern, such as antibiotic resistant bacteria and genes, in the reclaimed water. Therefore, this thesis performed an assessment of the presence, persistence and characterization of last-line antibiotic resistant bacteria and genes, from the produced wastewater influents to the reused streams, in two Portuguese full-scale wastewater treatment plants, and the application of an additional nanofiltration treatment step to test its removal efficiencies of these antibiotic-related pollutants for the subsequent production of a high-quality effluent that could be more safely reused in agricultural irrigation.

1.6 AIM AND OUTLINE OF THIS THESIS

The aim of the work developed and presented in this thesis is: (1) To provide a deep insight on the occurrence and persistence of the carbapenem resistome, as well as on the characterization of the corresponding resistant bacteria, from the produced wastewater influents to the reused streams, in two Portuguese full-scale wastewater treatment plants; (2) To address if the application of a final nanofiltration treatment step can be considered an alternative to produce high-quality wastewater effluents, free of antibiotic-related pollutants, that could be more safely reused, for example, in agricultural irrigation.

This thesis is divided into five chapters.

Chapter 1 consists in a revision of the state-of-the-art on the question of water scarcity, on how wastewater reuse can help to mitigate this problem and on the main challenges that this practice still raises, being the presence of emerging contaminants, such as antibiotic resistant bacteria and genes, in the wastewater effluents one of the main issues to be addressed. The conventional process by which wastewater is often treated worldwide is presented and the advanced wastewater treatments that can be applied to produce effluents with superior quality are also discussed.

In Chapter 2, the population dynamics of two full-scale wastewater treatment plants was characterized along different sampling points, including the discharged and reused effluents, in both cellular and extracellular DNA fractions. The analyses were performed by high throughput sequencing targeting the 16S rRNA V4 gene region and by three developed and optimized *TaqMan* multiplex qPCR assays that detect and quantify the most clinically relevant and globally distributed carbapenem and (fluoro)quinolone resistance genes (Oliveira et al., 2020). To the best of the authors' knowledge, this was the first study to undertake a comprehensive analysis on the concentrations of both carbapenem and (fluoro)quinolone resistance genes in the extracellular fraction of wastewaters.

In Chapter 3, two distinct groups of carbapenem resistant bacteria – potentially environmental and potentially pathogenic – were isolated from both the wastewater influent and discharged effluent samples of a full-scale wastewater treatment plant and subsequently genotypically and phenotypically characterized by whole genome sequencing and antibiotic susceptibility testing, respectively (Oliveira et al., 2021).

In Chapter 4, the potential of a commercial Desal 5 DK nanofiltration membrane to be used as a tertiary treatment step in the wastewater treatment plants for a more effective elimination of both antibiotic resistant bacteria and genes from the treated wastewater effluents was assessed on laboratory scale using a stainless steel cross-flow cell (Oliveira et al., 2022).

In Chapter 5, the main results obtained in each chapter are summarized in an overall discussion of the work presented in this thesis. Future work and perspectives are also presented.

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CHAPTER 2

The environmental contribution to the dissemination of carbapenem and (fluoro)quinolone resistance genes by discharged and reused wastewater effluents: The role of cellular and extracellular DNA

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Micaela Oliveira was involved in all the experimental work presented in this chapter, with exception of the high throughput sequencing and corresponding bioinformatic processing, which were performed by DNASense ApS. Mónica Nunes, Maria Teresa Barreto Crespo and Ana Filipa Silva supervised all the experimental work performed.

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ABSTRACT

Wastewater treatment plants (WWTPs) are major reservoirs and sources for the dissemination of antibiotic resistance into the environment. In this study, the population dynamics of two full-scale WWTPs was characterized along different sampling points, including the reused effluents, in both cellular and extracellular DNA samples. The analysis was performed by high throughput sequencing targeting the 16S rRNA V4 gene region and by three in-house *TaqMan* multiplex qPCR assays that detect and quantify the most clinically relevant and globally distributed carbapenem (*bla*) and (fluoro)quinolone (*qnr*) resistance genes. The obtained results identify the biological treatment as the crucial step on tailoring the wastewater bacterial community, which is thereafter maintained in both discharged and reused effluents. The influent bacterial community does not alter the WWTP core community, although it clearly contributes for the introduction and spread of antibiotic resistance to the in-house bacteria. The presence of high concentrations of *bla* and *qnr* genes was not only detected in the wastewater influents and discharged effluents, but also in the reused effluents, which therefore represent another gateway for antibiotic resistant bacteria and genes into the environment and directly to the human populations. Moreover, and together with the study of the cellular DNA, it was described, for the first time, the role of the extracellular DNA in the dissemination of carbapenem and (fluoro)quinolone resistance, as well as the impact of the wastewater treatment process on this DNA fraction. Altogether, the results prove that the current wastewater treatments are inefficient in the removal of antibiotic resistant bacteria and genes and reinforce that targeted treatments must be developed and implemented at full-scale in the WWTPs for wastewater reuse to become a safe and sustainable practice, able to be implemented in areas such as agricultural irrigation.

Keywords: Antibiotic resistance; Carbapenems; (Fluoro)quinolones; Wastewater treatment plants; Reused wastewater; Extracellular DNA

2.1 INTRODUCTION

In the last decades, antibiotics (ABs) have been increasingly used in human and veterinary medicine, as well as in other areas, such as agriculture, livestock and aquaculture. Considerable fractions of these pharmaceuticals are excreted into sewage in their unmetabolized original forms or as active and inactive metabolites, ending up in the wastewater treatment plants (WWTPs) (Jelic et al., 2011). Therefore, the bacterial communities present in the WWTPs are continuously exposed to sub inhibitory concentrations of different ABs, which promotes the selection and spread of resistances (Davies et al., 2006). Moreover, and since the conventional wastewater treatments are not designed for the removal of AB resistant bacteria and genes, WWTPs are considered major anthropogenic reservoirs and sources for the dissemination of these pollutants into the environment (Guo et al., 2017; Michael et al., 2013; Rizzo et al., 2013). The presence of AB resistant bacteria and genes towards the most commonly used classes of ABs have been extensively reported in the influents and effluents of several WWTPs worldwide (Cacace et al., 2019; Mao et al., 2015; Munir et al., 2011; Rafraf et al., 2016). However, the resistance towards last-line ABs, such as carbapenems, has only started to be assessed in the wastewaters more recently, and further studies are still required to fully understand not only the occurrence of these resistant bacteria and genes in the distinct geographic locations, but also their fate along the most critical steps of different wastewater treatment processes.

Carbapenems are currently one of the most important classes of ABs used to treat severe and persistent multiresistant infections caused by both Gram-positive and Gram-negative bacteria (Kattan et al., 2008; Papp-Wallace et al., 2011). Nevertheless, their efficiency is threatened by the global emergence and spread of carbapenem resistant bacteria, which harbour carbapenem resistance genes that express carbapenemases. Carbapenemases are a particular group of β-lactamases that inactivate carbapenems and other β-lactam ABs (Papp-Wallace et al., 2011; Poirel et al., 2007; Walsh, 2010). Regarding their clinical relevance and global distribution, the

most important carbapenemases are: (1) The class A serine-β-lactamases of the KPC type; (2) The class B metallo-β-lactamases of the NDM, IMP and VIM types; (3) The class D serine-β-lactamases of the OXA-48 type (Nordmann et al., 2012; Pfeifer et al., 2010). As with other β-lactamases, the majority of the carbapenemase-encoding genes are located in conjugative plasmids, which makes them easily transferable between bacteria and favors their fast and wide distribution (Nordmann et al., 2012; Pfeifer et al., 2010; Schultsz and Geerlings, 2012). The co-occurrence of carbapenem and (fluoro)quinolone resistance genes in the same plasmids is common (Schultsz and Geerlings, 2012; Strahilevitz et al., 2009). In fact, the most relevant (fluoro)quinolone resistance genes, which correspond to the *qnr*A, *qnr*B and *qnr*S-type genes, have multiple variants identified and numerous reports of co-localization with carbapenemase-encoding genes in conjugative plasmids, leading to their simultaneous transfer even when bacteria are only pressured by one AB (Schultsz and Geerlings, 2012; Strahilevitz et al., 2009). These (fluoro)quinolone resistance genes have already been identified and are often present along the different steps of several wastewater treatment processes worldwide (Auguet et al., 2017; Mao et al., 2015; Neudorf et al., 2017; Rafraf et al., 2016). However, little is still known about their likely co-occurrence with carbapenem resistance genes in such environments.

Recent studies have reported the detection of both carbapenem resistant bacteria and genes in the wastewater influents and discharged effluents of different WWTPs of Europe (Auguet et al., 2017; Cacace et al., 2019; Pärnänen et al., 2019), Asia (Lamba and Ahammad, 2017; Yang et al., 2016) and Northern America (Mathys et al., 2019; Naquin et al., 2017). Nevertheless, there are still major gaps in the current knowledge that have to be overcome to better demonstrate the importance of the environmental sector in the global resistance to both carbapenems and (fluoro)quinolones: (1) The search for all main carbapenem resistance genes, to provide a more accurate picture regarding the carbapenem resistome present in the WWTPs; (2) The look for the possible co-occurrence of carbapenem and (fluoro)quinolone resistance genes, since

the resistance to these two classes of ABs is often simultaneously transferred between bacteria; (3) The analysis of reused wastewater samples, which can represent an additional gateway for AB resistant bacteria and genes into the environment and directly to the human populations; (4) The focus not only on the cellular DNA, but also on the free / extracellular DNA (eDNA), which can harbour an important fraction of AB resistance genes that are able to be assimilated by non-resistant bacteria via natural transformation. This study addresses these questions and provides a deep insight on the occurrence and characterization of the carbapenem and (fluoro)quinolone resistome, from the anthropogenic produced wastewater influents to the reused streams, in two Portuguese full-scale WWTPs. To achieve such goal, the main objectives are:

1) Characterize the bacterial community composition along different sampling points of the two WWTPs by high throughput sequencing targeting the 16S rRNA V4 gene region.

2) Investigate the prevalence and fate of carbapenem and (fluoro)quinolone resistance genes in DNA and eDNA samples using three in-house *TaqMan* multiplex qPCR assays.

3) Assess whether the different designs and operational conditions of the two WWTPs impact differently the removal of carbapenem and (fluoro)quinolone resistant bacteria and genes, preventing them from reaching the environment and / or directly the human populations by wastewater reuse.

2.2 MATERIALS AND METHODS

2.2.1 WWTPs design description and sample collection

Two Portuguese full-scale WWTPs from the metropolitan Lisbon area were selected for this study: (1) The WWTP A was designed to treat the domestic wastewater of approximately 756,000 population equivalents (P.E.) and employs a biological

treatment step with biological aerated filters (BAF). Most of the produced effluent is discharged into the Tagus River and a smaller fraction is filtered through a cartridge filter, disinfected with the addition of sodium hypochlorite and then reused for green park irrigation and street washing purposes. (2) The WWTP B was designed to treat the domestic wastewater of approximately 211,000 P.E. and employs the conventional biological nutrient removal by activated sludge (BNR AS). After the biological treatment step, the wastewater is filtered through a sand filter, being most of the produced effluent discharged into the Tagus River. The remaining effluent is disinfected with the addition of sodium hypochlorite and then reused for green park irrigation and street washing purposes. For each WWTP, four sampling points were defined and a total sample of 30 L (consisting in three biological replicates of 10 L) was collected from each one in sterile containers on April 9, 2019. The samples were transported to the laboratory under refrigerated conditions and immediately processed upon arrival. The main steps of both wastewater treatment processes and the four sampling points defined for each WWTP are shown in Figure 2.1.

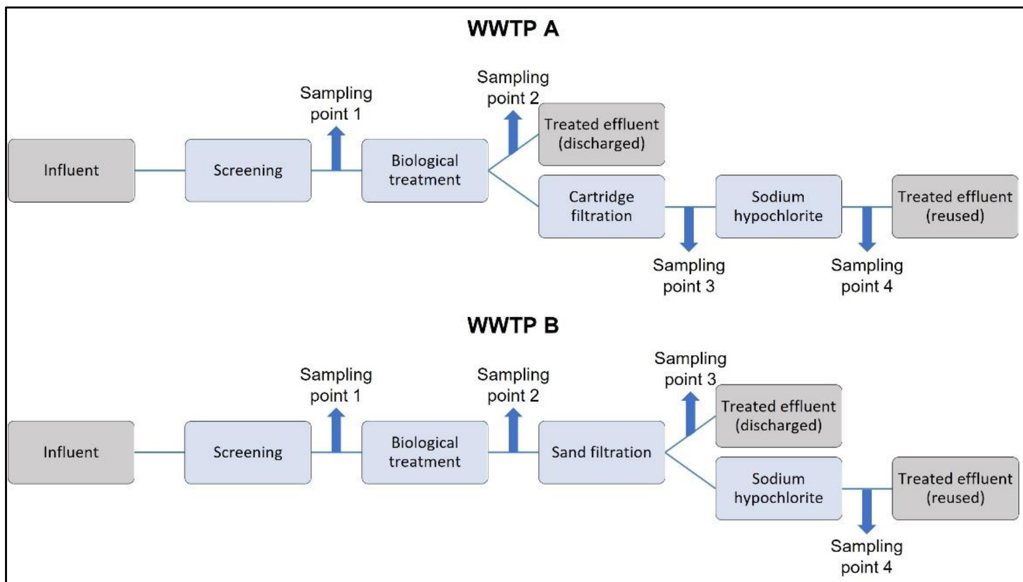

Figure 2.1 | Main steps of the wastewater treatment process and sampling points defined for each WWTP.

2.2.2 Bacterial community analysis targeting the 16S rRNA V4 gene region

2.2.2.1 DNA extraction

The wastewater samples (n = 8) were filtered in triplicate through 0.22 µm pore-size polycarbonate filters (Whatman, Maidstone, UK) and the DNA was extracted from the filters using the standard protocol from the DNeasy® PowerWater® Kit (Qiagen, Hilden, Germany). DNA concentrations were measured on a Qubit Fluorometer using the Qubit dsDNA HS/BR Assay Kit (Thermo Fisher Scientific, Waltham, USA) and are shown in Table S2.1.

2.2.2.2 Library preparation

The 16S rRNA V4 gene region sequencing libraries were prepared by a custom protocol based on an Illumina protocol (Illumina, 2015). Up to 10 ng of each extracted DNA were used as template for PCR amplification of the 16S rRNA V4 gene region. Each PCR reaction (25 µL) contained 12.5 µL PCRBIO Ultra Mix (PCR Biosystems, London, UK) and 400 nM of each forward and reverse tailed primers. The PCR reactions were conducted using the following program: Initial denaturation at 95 °C for 2 min; 30 cycles of amplification at 95 °C for 15 s, 55 °C for 15 s and 72 °C for 50 s; final elongation at 72 °C for 5 min. Duplicate PCR reactions were performed for each sample and the duplicates were pooled after the PCR. The forward and reverse tailed primers were designed according to (Illumina, 2015) and contain primers targeting both the archaeal and bacterial 16S rRNA V4 gene region: [515F] GTGYCAGCMGCCGCGGTAA and [806R] GGACTACNVGGGTWTCTAAT (Apprill et al., 2015). The primer tails enable the attachment to the Illumina Nextera adaptors necessary for sequencing in a subsequent PCR. The resulting amplicon libraries were purified using the standard protocol for Agencourt Ampure XP Beads (Beckman Coulter, Brea, USA) with a bead to sample ratio of 4:5 and the DNA was eluted in 25 µL of nuclease free water (Qiagen, Hilden, Germany). DNA concentrations were measured using the Qubit dsDNA HS Assay Kit

(Thermo Fisher Scientific, Waltham, USA) and a gel electrophoresis using Tapestation 2200 and D1000 / High sensitivity D1000 screentapes (Agilent, Santa Clara, USA) was used to validate the product size and purity of a subset of the purified amplicon libraries. The sequencing libraries were prepared from the purified amplicon libraries using a second PCR. Each PCR reaction (25 µL) contained PCRBIO HiFi buffer (1x), PCRBIO HiFi Polymerase (1 U / reaction) (PCR Biosystems, London, UK), adaptor mix (400 nM of each forward and reverse) and up to 10 ng of amplicon library template. The PCR reactions were conducted using the following program: Initial denaturation at 95 °C for 2 min, 8 cycles of amplification at 95 °C for 20 s, 55 °C for 30 s and 72 °C for 60 s; final elongation at 72 °C for 5 min. The resulting sequencing libraries were purified using the standard protocol for Agencourt Ampure XP Beads (Beckman Coulter, Brea, USA) with a bead to sample ratio of 4:5 and the DNA was eluted in 25 µL of nuclease free water (Qiagen, Hilden, Germany). DNA concentrations were measured using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, USA) and a gel electrophoresis using Tapestation 2200 and D1000/High sensitivity D1000 screentapes (Agilent, Santa Clara, USA) was used to validate the product size and purity of a subset of the purified sequencing libraries.

2.2.2.3 DNA sequencing

The purified sequencing libraries were pooled in equimolar concentrations and diluted to 2 nM. The samples were paired-end sequenced (2 x 300 bp) on a MiSeq (Illumina, San Diego, USA), using a MiSeq Reagent Kit v3 (Illumina, San Diego, USA) and following the standard guidelines for preparing and loading samples on the MiSeq. >10 % PhiX control library was spiked in to overcome low complexity issues often observed with amplicon samples.

2.2.2.4 Bioinformatic processing

Forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32 (Bolger et al., 2014) with the settings SLIDINGWINDOW:5:3 and MINLEN:225. The trimmed forward and reverse reads were merged using FLASH v. 1.2.7 (Magoč and Salzberg, 2011) with the settings -m 10 -M 250. The merged reads were dereplicated and formatted for use in the UPARSE workflow (Edgar, 2013). The dereplicated reads were clustered using the usearch v. 7.0.1090 -cluster_otus command with default settings. OTU abundances were estimated using the usearch v. 7.0.1090 -usearch_global command with -id 0.97 -maxaccepts 0 -maxrejects 0. Taxonomy was assigned using the RDP classifier (Wang et al., 2007), as implemented in the parallel_assign_taxonomy_rdp.py script in QIIME (Caporaso et al., 2010), using -confidence 0.8 and the SILVA database, release 132 (Quast et al., 2013). The results were analyzed in R v. 3.5.1 (R Core Team, 2017) through the Rstudio IDE, using the ampvis package v.2.4.5 (Albertsen et al., 2015). The sequence data files were deposited in GenBank within the BioProject with the SRA accession number PRJNA599309. Both the high throughput sequencing and the bioinformatic processing were conducted by DNASense ApS (Aalborg, Denmark).

2.2.3 Detection and quantification of carbapenem and (fluoro)quinolone resistance genes by *TaqMan* multiplex qPCR

2.2.3.1 DNA and eDNA extraction

The wastewater samples (n = 8) were primarily filtered in triplicate through 0.45 µm pore-size polyethersulfone filters (Pall Corporation, New York, USA) and the resulting filtrates were again filtered through 0.22 µm pore-size polyethersulfone filters (Pall Corporation, New York, USA). Filtration volumes were defined by clogging of the filters as a measure of the same amount of filtered biomass. Therefore, volumes of 30 mL for WWTP A and 35 mL for WWTP B from sampling point 1, 90 mL for WWTP A and

350 mL for WWTP B from sampling point 2, 200 mL for WWTP A and 550 mL for WWTP B from sampling point 3 and 250 mL for WWTP A and 600 mL for WWTP B from sampling point 4 were filtered. After filtration, the filters proceeded for DNA extraction and 15 mL of each final filtrate proceeded for precipitation and purification of the eDNA. The DNA was extracted from each of the two filters per sample following the standard protocol from the DNeasy® PowerWater® Kit (Qiagen, Hilden, Germany), being recovered in 50 µL elution buffer. At the end, the DNAs extracted from both related filters were mixed together into one final DNA. For the eDNA, the final filtrates were precipitated with absolute ethanol and 3 M sodium acetate, as previously described by (Foote et al., 2012), and purified using the DNeasy® UltraClean® Microbial Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA and eDNA concentrations and purity were measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and are shown in Tables S2.2 and S2.3.

2.2.3.2 Development and optimization of the *TaqMan* multiplex qPCR assays

According to their importance in terms of clinical relevance and global distribution, five carbapenem – *bla*$_{KPC}$, *bla*$_{OXA\text{-}48}$, *bla*$_{NDM}$, *bla*$_{IMP}$ and *bla*$_{VIM}$ – and three (fluoro)quinolone – *qnr*A, *qnr*B and *qnr*S – resistance genes were chosen for this study. In order to rapidly detect and quantify them, three *TaqMan* multiplex qPCR assays were developed and optimized: (1) *TaqMan* multiplex qPCR for the detection and quantification of the carbapenemase-encoding genes of the *bla*$_{KPC}$ and *bla*$_{OXA\text{-}48}$ types; (2) *TaqMan* multiplex qPCR for the detection and quantification of the carbapenemase-encoding genes of the *bla*$_{NDM}$, *bla*$_{IMP}$ and *bla*$_{VIM}$ types; (3) *TaqMan* multiplex qPCR for the detection and quantification of the (fluoro)quinolone resistance genes of the *qnr*A, *qnr*B and *qnr*S types. Additionally, a *TaqMan* qPCR assay for the detection and quantification of the 16S rRNA gene was also developed and optimized, in order to assess the bacterial abundance in the different wastewater samples. The sequences of

the primers and probes used in these *TaqMan* qPCR reactions were retrieved from the literature or designed in the course of this study. For that, multiple alignments of reference sequences retrieved from GenBank and ResFinder (Zankari et al., 2012) for each target gene were executed on Mafft 7 (Katoh and Standley, 2013) and the design of the primers and probes was performed using a combination of tools, including Multiple Primer Analyzer (Thermo Fisher Scientific, Waltham, USA) and PrimerBlast (Ye et al., 2012). Information about the primers and probes is provided in Table 2.1. All *TaqMan* qPCR reactions were developed and optimized according to the MIQE guidelines (Bustin et al., 2009), being initially performed with different primer and probe concentrations, as well as different amplification conditions, in order to find the optimal combinations of these variables. Also, for each *TaqMan* qPCR, the specificity of the reaction was confirmed using the DNA of the other resistance genes under study as templates.

Table 2.1 | Information about the sequence, amplicon size and bibliographic reference of the primers and probes used in each *TaqMan* qPCR reaction.

Gene	Primer / Probe	Sequence (5' – 3')	Amplicon (bp)	Reference
bla_{KPC}	KPC Fw	GACGGAAAGCTTACAAAAACTGACA	259	This study
	KPC Rv	CTTGTCATCCTTGTTAGGCG		(Poirel et al., 2011)
	KPC Probe	FAM-ACTGGGCAGTCGGAGACAAAACCGGA-BHQ1		This study
bla_{OXA-48}	OXA-48 Fw	TTCGAATTTCGGCCACGG	204	This study
	OXA-48 Rv	CATCAAGTTCAACCCAACCG		(Poirel et al., 2011)
	OXA-48 Probe	HEX-CCATGCTGACCGAAGCCAATGGTG-BHQ1		This study
bla_{NDM}	NDM Fw	GGTTTGGCGATCTGGTTTTC	181	(Poirel et al., 2011)
	NDM Rv	ATCCAGTTGAGGATCTGGGC		(Swayne et al., 2013)
	NDM Probe	FAM-CGGGGCAGTCGCTTCCAACGGTT-BHQ1		This study
bla_{IMP}	IMP Fw	GGAATAGAGTGGCTTAAYTCTC	275	(Österblad et al., 2012)
	IMP Rv	CAAGCTTCTATATTTGCGTCACC		This study
	IMP Probe	HEX-TTATCCAGGCCCGGGACACAC-BHQ1		This study
bla_{VIM}	VIM Fw	GATGAGTTGCTTTTGATTGATACAGC	153	(Swayne et al., 2013)
	VIM Rv	CGCCCGAAGGACATCAA		(Favaro et al., 2014)
	VIM Probe	ROX-ACGCACTTTCATGACGACCGCGTC-BHQ2		(Favaro et al., 2014)
qnrA	qnrA Fw	GGATGCCAGTTTCGAGGA	154	(Cavaco et al., 2008)
	qnrA Rv	CCTGAACTCTATGCCAAAGC		(Vien et al., 2012)
	qnrA Probe	FAM-CACTTCAGCTATGCCGATCTGCGCGAT-BHQ1		This study
qnrB	qnrB Fw	CAGATTTYCGCGGCGCAAG	134	(Vien et al., 2012)
	qnrB Rv	TTCCCACAGCTCRCAYTTTTC		
	qnrB Probe	HEX-CGCACCTGGTTTTGYAGYGCMTATATCAC-BHQ1		
qnrS	qnrS Fw	GCCCATCAAGTGAGTAATCGTATG	293	This study
	qnrS Rv	CAGGCTGCAATTTTGATACCT		
	qnrS Probe	ROX-AACGAACCTAGCGGGTGCATCACTG-BHQ2		
16S rRNA	16S Fw	GAATGCCACGGTGAATACGTT	157	(Lacour and Landini, 2004)
	16S Rv	TCCCTACGGTTACCTTGTTACG		This study
	16S Probe	FAM-CACACCGCCCGTCACACCATGGGAG-BHQ1		This study

2.2.3.3 Generation of the standard curves for the *TaqMan* qPCR assays

To generate the standard curves for each *TaqMan* qPCR, fragments of the target genes were amplified by conventional PCR. The sequences of the primers used in the PCR reactions were retrieved from the literature or designed in the course of this study, as described in section 2.2.3.2. Information about the primers is provided in Table 2.2. Each PCR reaction (25 µL) contained 12.5 µL NZYTaq II 2x Green MasterMix (NZYTech, Lisbon, Portugal), 200 nM of each forward and reverse primers, 50 ng of DNA template and nuclease free water (to complete 25 µL). The PCR reactions were conducted using the following program: Initial denaturation at 95 °C for 3 min; 35 cycles of amplification at 94 °C for 30 s, annealing temperature for 30 s and 72 °C for 45 s; final elongation at 72 °C for 5 min. The annealing temperatures were 57 °C for the 16S rRNA, bla_{KPC} and bla_{OXA-48}-type genes, 60 °C for bla_{NDM}, bla_{IMP} and bla_{VIM}-type genes and 58 °C for *qnr*A, *qnr*B and *qnr*S-type genes. The PCR products were then purified using the NZYGelpure Kit (NZYTech, Lisbon, Portugal), inserted into pGEM-T Easy Vector Systems (Promega, Madison, USA) and transformed into JM109 competent cells (Promega, Madison, USA), according to the manufacturer's instructions. The selection was based on the inactivation of β-galactosidase and the recombinant clones were distinguished by blue / white colonies. All (white) positive clones for each target gene were selected and plasmids were extracted using the standard protocol from the NZYMiniprep Kit (NZYTech, Lisbon, Portugal). Sanger sequencing was performed to ensure the specificity and correct insertion of the purified PCR products in the corresponding plasmids. Plasmid DNA concentrations and purity were measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, USA). Gene copy numbers were calculated as previously described by (Ritalahti et al., 2006) and standard curves for each target gene were generated from tenfold dilutions of the extracted plasmids, ranging from 10^7 to 10^0 gene copy numbers / µL, which allowed the assessment of the efficiency and sensitivity of each *TaqMan* qPCR.

Table 2.2 | Information about the sequence, amplicon size and bibliographic reference of the primers used in each PCR reaction.

Gene	Primer	Sequence (5' – 3')	Amplicon (bp)	Reference
bla$_{KPC}$	KPC Fw	CGTCTAGTTCTGCTGTCTTG	804	(Poirel et al., 2011)
	KPC Rv	GCTGTGCTTGTCATCCTTGTT		This study
bla$_{OXA-48}$	OXA-48 Fw	AATGCCTGCGGTAGCAAAGG	678	This study
	OXA-48 Rv	GTGGGCATATCCATATTCATCGCA		
bla$_{NDM}$	NDM Fw	CATTAGCCGCTGCATTGATG	708	(Van der Zee et al., 2014)
	NDM Rv	CGGAATGGCTCATCACGATC		(Poirel et al., 2011)
bla$_{IMP}$	IMP Fw	GCATTGCTACCGCAGCAGAGTC	670	This study
	IMP Rv	TCGTTTAACCCTTTAACCGCCTGC		
bla$_{VIM}$	VIM Fw	CAGATTGCCGATGGTGTTTGG	615	(Lombardi et al., 2002)
	VIM Rv	TGTGTGCTTGAGCAAGTCTAGA		This study
*qnr*A	qnrA Fw	AGAGGATTTCTCACGCCAGG	619	(Cattoir et al., 2007b)
	qnrA Rv	CAGCACTATTACTCCCAAGGGT		This study
*qnr*B	qnrB Fw	GGMATHGAAATTCGCCACTG	263	(Cattoir et al., 2007a)
	qnrB Rv	TTTGCYGYYCGCCAGTCGAA		
*qnr*S	qnrS Fw	CGGCACCACAACTTTTCACATAAAG	628	This study
	qnrS Rv	CAGGATAAACAACAATACCCAGTGC		
16S rRNA	16S Fw	GCCAGCAGCCGCGGTAA	1024	Adapted from
	16S Rv	AAGGAGGTGATCCRGCCGCA		(Turner et al., 1999)

2.2.3.4 Detection and quantification of the target carbapenem and (fluoro)quinolone resistance genes by *TaqMan* multiplex qPCR

The quantification of the target carbapenem (*bla*) and (fluoro)quinolone (*qnr*) resistance genes, as well as of the 16S rRNA gene, was conducted in triplicate on a LightCycler 96 Real-Time PCR System (Roche, Basel, Switzerland) using the following program: DNA denaturation / polymerase activation at 95 °C for 5 min; 40 cycles of amplification at 95 °C for 10 s and 60 °C for 30 s. Information about the mix reactions of each *TaqMan* qPCR is provided in Table 2.3.

Table 2.3 | Information about the concentrations and volumes of mastermix, primers, probes, DNA / eDNA templates and nuclease free water used in each *TaqMan* qPCR reaction.

***TaqMan* qPCR**	**Mix reaction**
TaqMan multiplex qPCR 1: bla_{KPC} and bla_{OXA-48}	10 µL SensiFAST Probe No-ROX Kit (Bioline, London, UK) 400 nM bla_{KPC} forward and reverse primers; 200 nM bla_{OXA-48} forward and reverse primers 100 nM bla_{KPC} and bla_{OXA-48} *TaqMan* probes 50 ng bla_{KPC} and bla_{OXA-48} DNA templates or 10 ng bla_{KPC} and bla_{OXA-48} eDNA templates Nuclease free water (to make 20 µL)
TaqMan multiplex qPCR 2: bla_{NDM}, bla_{IMP} and bla_{VIM}	10 µL SensiFAST Probe No-ROX Kit (Bioline, London, UK) 200 nM bla_{NDM}, bla_{IMP} and bla_{VIM} forward and reverse primers 100 nM *bla*NDM and *bla*IMP *TaqMan* probes; 10 nM bla_{VIM} *TaqMan* probe 50 ng bla_{NDM}, bla_{IMP} and bla_{VIM} DNA templates or 10 ng bla_{NDM}, bla_{IMP} and bla_{VIM} eDNA templates Nuclease free water (to make 20 µL)
TaqMan multiplex qPCR 3: *qnr*A, *qnr*B and *qnr*S	10 µL SensiFAST Probe No-ROX Kit (Bioline, London, UK) 200 nM *qnr*A, *qnr*B and *qnr*S forward and reverse primers 100 nM *qnr*A and *qnr*B *TaqMan* probes; 10 nM *qnr*S *TaqMan* probe 50 ng *qnr*A, *qnr*B and *qnr*S DNA templates or 10 ng *qnr*A, *qnr*B and *qnr*S eDNA templates Nuclease free water (to make 20 µL)
TaqMan singleplex qPCR: 16S rRNA	10 µL SensiFAST Probe No-ROX Kit (Bioline, London, UK) 400 nM 16S rRNA forward and reverse primers 100 nM 16S rRNA *TaqMan* probe 50 ng 16S rRNA DNA template or 10 ng 16S rRNA eDNA template Nuclease free water (to make 20 µL)

2.2.4 Statistical analysis

A principal component analysis (PCA) was performed to identify the samples with similar bacterial community compositions. Additionally, several one-way analysis of variance tests (ANOVA) were conducted to compare the mean values of both the bacterial community composition and the target carbapenem and (fluoro)quinolone resistance genes along the different sampling points of each WWTP and / or between the corresponding sampling points of both WWTPs. Different methodologies were adopted, depending on whether the variances were homogeneous or not. The homogeneity of the variances was previously evaluated with different Levene's Tests

(Tables S2.4, S2.5 and S2.6): (1) If the resulting differences were significant, the variances were not considered homogeneous and an ANOVA with the Dunnett T3 post hoc test was performed; (2) If the resulting differences were not significant, the variances were considered homogeneous and an ANOVA with the Tukey post hoc test was performed. These statistical analyses were performed using the SPSS 26 software (IBM, Armonk, USA) and the differences were considered significant at $p < 0.05$.

2.3 RESULTS

2.3.1 Bacterial community composition of the wastewater samples

According to the PCA performed, the bacterial community composition of both wastewater influents was similar (Figure 2.2). However, the impact of the two distinct biological treatments resulted in the formation of two bacterial communities that were simultaneously different from the respective influent samples and from each other (Figure 2.2).

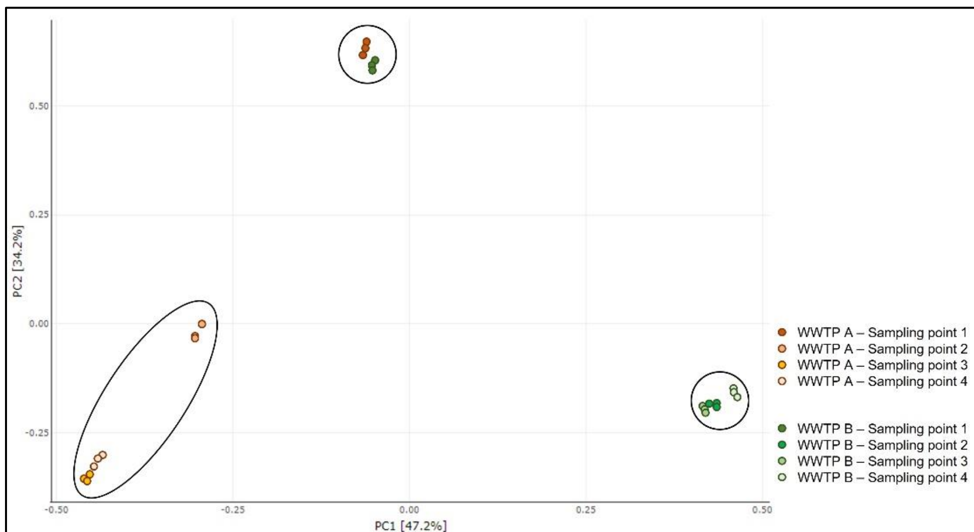

Figure 2.2 | Identification of the samples with a similar bacterial community composition by a principal component analysis (PCA). The relative contribution (eigenvalue) of each axis to the total inertia in the data is expressed in percentage at each axis title.

Regarding a higher taxonomic level, the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria were predominant in all sampling points of the two WWTPs (Figure 2.3a; Table S2.7). Nevertheless, significant differences ($p < 0.05$) in their relative abundances were observed not only between the corresponding sampling points of both WWTPs, but also after the biological treatment of each WWTP (Figure 2.3a; Tables S2.7, S2.8 and S2.9). In WWTP A, the decrease in the relative abundance of the Firmicutes, from 45.26 % to 9.73 %, was simultaneous to the increase in the relative abundances of the phyla Bacteroidetes, from 2.42 % to 30.51 %, and Proteobacteria, from 36.62 % to 55.07 % (Figure 2.3a; Table S2.7). In WWTP B, the decrease in the relative abundances of the Firmicutes, from 33.76 % to 12.33 %, and Proteobacteria, from 51.37 % to 39.16 %, was simultaneous to the increase in the relative abundance of the phylum Actinobacteria, from 5.19 % to 19.75 % (Figure 2.3a; Table S2.7). At a lower taxonomic level, bacteria belonging to several genera associated with the human intestinal microbiota, such as *Bifidobacterium*, *Blautia*, *Comamonas*, *Faecalibacterium* and *Jeotgalibaca* were well represented in the wastewater influents of the two WWTPs, despite being efficiently outcompeted or retained at the biological treatment step (Figure 2.3b; Table S2.10). Bacteria belonging to genera associated with potential opportunistic pathogens were also detected with high relative abundances in the influents of both WWTPs (Figure 2.3b; Table S2.10). In fact, the genus *Acinetobacter* was the most represented of all genera in the influents of the two WWTPs, with a relative abundance of 13.42 % in WWTP A and 26.75 % in WWTP B, being also detected in the discharged effluent of WWTP A, with a relative abundance of 5.52 %, and along the remaining sampling points of WWTP B, with relative abundances of 3.23 % in sampling point 2, 2.85 % in sampling point 3 and 2.54 % in sampling point 4 (Figure 2.3b; Table S2.10). Moreover, the genera *Moraxella* and *Streptococcus* were found in both wastewater influents, with relative abundances of 6.14 % and 7.57 %, respectively, in WWTP A and 3.32 % and 3.36 %, respectively, in WWTP B. Also, the genus *Arcobacter* was found along all sampling points of WWTP B, with relative abundances ranging from

4.25 % to 5.98 % (Figure 2.3b; Table S2.10). After the biological treatment step, bacteria from the genus *Pseudomonas* were found in the reused effluent of WWTP A, with a relative abundance of 3.40 % and, besides the previously mentioned genera *Acinetobacter* and *Arcobacter*, bacteria belonging to the genera *Bacteroides*, *Clostridium*, *Corynebacterium* and *Mycobacterium* were found in the discharged and reused effluents of WWTP B, with relative abundances of 1.58 %, 2.49 %, 1,03 % and 1.61 %, respectively, in the discharged effluent and 2.97 %, 3.78 %, 1.67 % and 1.78 %, respectively, in the reused effluent (Figure 2.3b; Table S2.10).

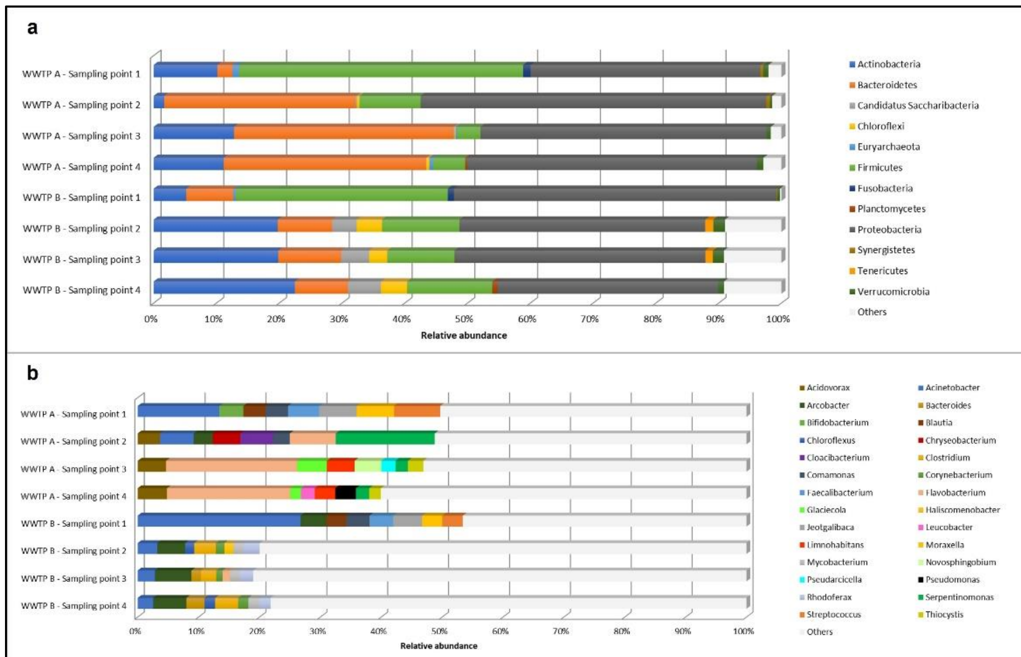

Figure 2.3 | Relative abundance of the eight most represented phyla (a) and genera (b) present in the different sampling points of both WWTPs. Values are expressed in percentages and correspond to the mean of biological triplicates.

2.3.2 Carbapenem and (fluoro)quinolone resistance genes present in the wastewater samples

For each *TaqMan* qPCR, standard curves were generated by tenfold dilutions of the templates, which allowed the estimation of the efficiency, coefficient of correlation (r^2) and sensitivity of the reactions (Table 2.4). All *TaqMan* qPCR reactions had amplification efficiencies between 86.80 % and 102.49 %, coefficients of correlation (r^2) between 0.9867 and 0.9999 and sensitivities of 10 gene copy numbers / µL (Table 2.4). The specificity of each qPCR reaction was also assessed, with no detection of any nonspecific amplification products within the set of templates used (Table S2.11).

Table 2.4 | Standard curve equations, amplification efficiencies, coefficients of correlation (r^2) and sensitivities of each *TaqMan* qPCR reaction. Values correspond to the mean of technical triplicates.

TaqMan qPCR	Genes	Standard curve equation	Amplification efficiency	Coefficient of correlation (r^2)	Sensitivity
TaqMan Singleplex qPCR	16S rRNA	y = -3.2636x + 38.647	102.49 %	0.9980	10 copies / µL
TaqMan Multiplex qPCR 1	bla_{KPC}	y = -3.4307x + 38.071	95.65 %	0.9988	10 copies / µL
	bla_{OXA-48}	y = -3.3568x + 36.476	98.57 %	0.9980	10 copies / µL
TaqMan Multiplex qPCR 2	bla_{NDM}	y = -3.6657x + 40.154	87.41 %	0.9964	10 copies / µL
	bla_{IMP}	y = -3.4320x + 40.041	95.60 %	0.9924	10 copies / µL
	bla_{VIM}	y = -3.3225x + 37.993	99.98 %	0.9867	10 copies / µL
TaqMan Multiplex qPCR 3	*qnr*A	y = -3.6850x + 39.703	86.80 %	0.9972	10 copies / µL
	*qnr*B	y = -3.5561x + 40.020	91.08 %	0.9999	10 copies / µL
	*qnr*S	y = -3.5357x + 37.153	91.79 %	0.9996	10 copies / µL

Regarding the study of the DNA extracted from the bacterial community cells, the quantification of the 16S rRNA gene was performed to assess the bacterial abundance in the different sampling points of the two WWTPs. The obtained concentrations ranged from 9.3×10^9 to 1.8×10^8 gene copy numbers / mL in WWTP A and from 6.6×10^9 to 2.9×10^7 gene copy numbers / mL in WWTP B (Figure 2.4; Table S2.12). In both WWTPs, a significant decrease ($p < 0.05$) of the bacterial abundance was observed after

the biological treatment step (Figure 2.4; Tables S2.12 and S2.13). Nevertheless, the 16S rRNA gene remained present at high concentrations in the discharged and reused effluents of the two WWTPs (Figure 2.4; Table S2.12). As for the AB resistance genes, seven out of the eight target carbapenem and (fluoro)quinolone resistance genes – bla_{KPC}, bla_{OXA-48}, bla_{NDM}, bla_{VIM}, *qnr*A, *qnr*B and *qnr*S – were detected at high concentrations in both wastewater influents (Figure 2.4; Table S2.12). The most abundant resistance genes in the influent of WWTP A were the *qnr*S and bla_{VIM}, with concentrations of 2.7 x 10^7 and 7.2 x 10^6 gene copy numbers / mL, respectively (Figure 2.4a; Table S2.12). In the influent of WWTP B, the most abundant resistance genes were the bla_{VIM} and bla_{OXA-48}, with concentrations of 9.4 x 10^5 and 4.0 x 10^5 gene copy numbers / mL, respectively (Figure 2.4b; Table S2.12). Despite the reduction in the average abundance of the target resistance genes observed in the two WWTPs after the biological treatment step, the bla_{KPC}, bla_{VIM}, *qnr*B and *qnr*S genes were detected along all sampling points of both WWTPs (Figure 2.4; Table S2.12). In fact, the discharged and reused effluents presented considerable concentrations of these genes, ranging from 5.2 x 10^5 to 4.6 x 10^4 gene copy numbers / mL in the discharged effluent of WWTP A, from 2.4 x 10^4 to 3.9 x 10^2 gene copy numbers / mL in the discharged effluent of WWTP B, from 6.9 x 10^4 to 6.9 x 10^3 gene copy numbers / mL in the reused effluent of WWTP A and from 2.8 x 10^4 to 7.2 x 10^2 gene copy numbers / mL in the reused effluent of WWTP B (Figure 2.4; Table S2.12). The unexpected single detection of the bla_{OXA-48} gene in the reused effluent of WWTP A was understood by us as a sporadic event that is not representative of this sampling point and that was probably due to a punctual development of a bacterial biofilm harbouring this resistance gene in the pipeline transporting the reused effluent (Figure 2.4a; Table S2.12).

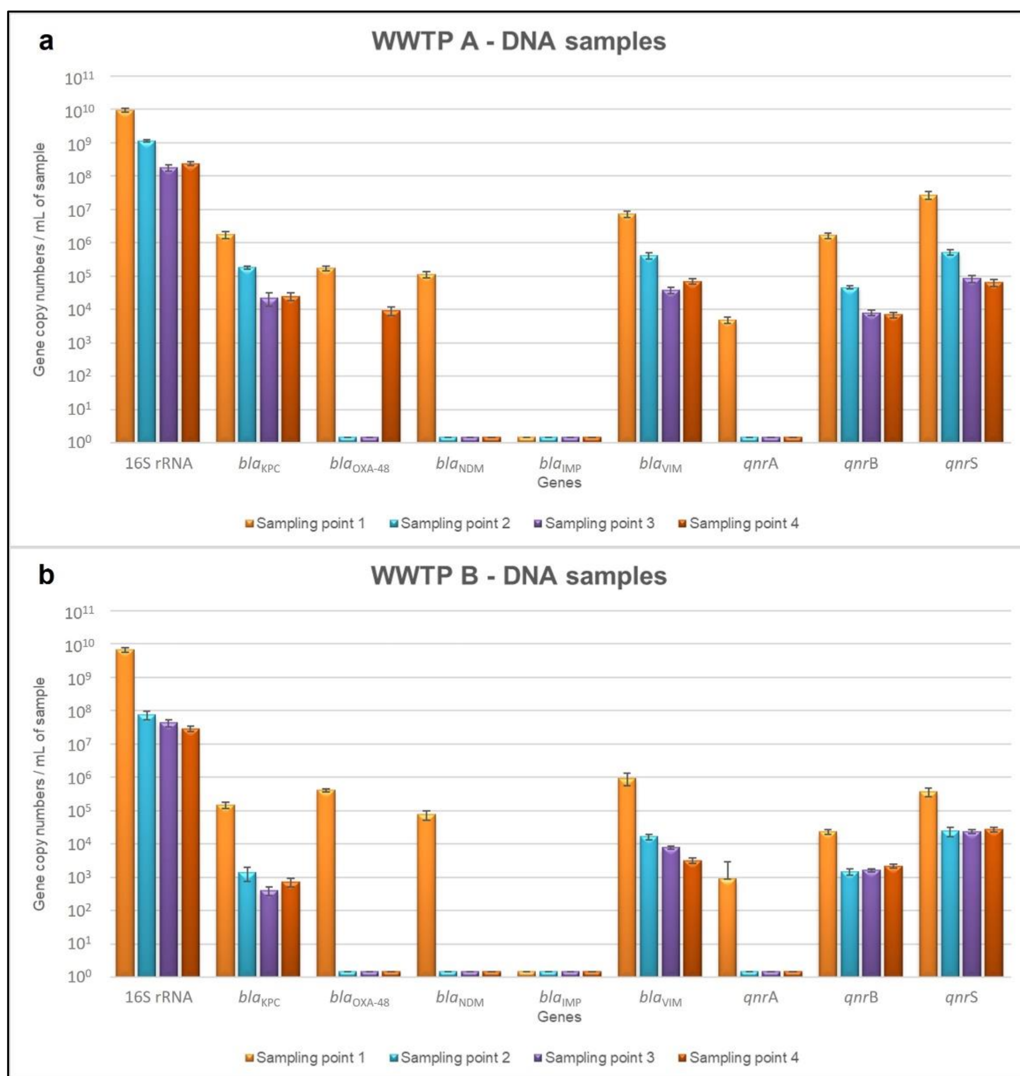

Figure 2.4 | Average abundance of the 16S rRNA, *bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *qnr*A, *qnr*B and *qnr*S genes in the DNA extracted from the bacterial community cells of the different sampling points of WWTP A (a) and WWTP B (b). Values are expressed in gene copy numbers per milliliter and correspond to the mean ± standard deviation of biological and technical triplicates.

In the study of the eDNA, the 16S rRNA gene was also detected along all sampling points of both WWTPs, at concentrations that ranged from 2.9 x 10^6 to 1.1 x 10^5 gene copy numbers / mL in WWTP A and from 1.7 x 10^6 to 2.7 x 10^4 gene copy numbers / mL

in WWTP B (Figure 2.5; Table S2.14). Contrary to the analysis of this gene in the DNA extracted from the bacterial community cells, in the eDNA samples these values do not represent bacterial abundance but free naked bacterial eDNA. In the influent of WWTP A, four out of the eight target carbapenem and (fluoro)quinolone resistance genes – *bla*$_{OXA-48}$, *bla*$_{VIM}$, *qnr*B and *qnr*S – were detected at concentrations ranging from1.2 x 10^5 to 2.6 x 10^2 gene copy numbers / mL (Figure 2.5a; Table S2.14). Three of these genes – *bla*$_{VIM}$, *qnr*B and *qnr*S – were detected along all sampling points, including the discharged and reused effluents, at concentrations that ranged from 3.9 x 10^3 to 3.9 x 10^2 gene copy numbers / mL in the discharged effluent and from 2.1 x 10^4 to 2.5 x 10^2 gene copy numbers / mL in the reused effluent (Figure 2.5a; Table S2.14). In the influent of WWTP B, five out of the eight target carbapenem and (fluoro)quinolone resistance genes – *bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{VIM}$, *qnr*B and *qnr*S – were detected at concentrations ranging from 1.0 x 10^4 to 5.7 x 10^2 gene copy numbers / mL (Figure 2.5b; Table S2.14). One of these genes – *bla*$_{VIM}$ – was detected along all sampling points, including the discharged and reused effluents, at a concentration of 5.3 x 10^3 gene copy numbers / mL in the discharged effluent and 3.2 x 10^3 gene copy numbers / mL in the reused effluent (Figure 2.5b; Table S2.14).

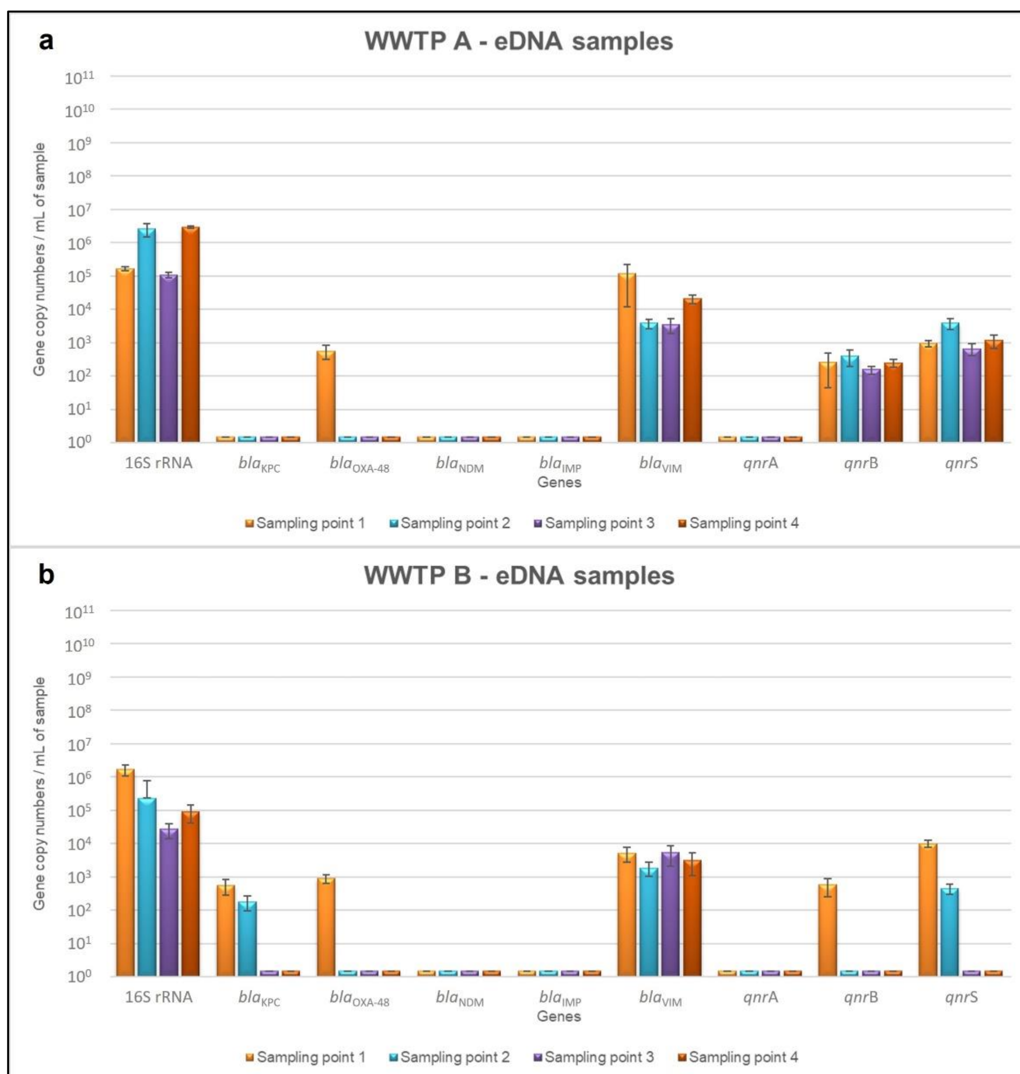

Figure 2.5 | Average abundance of the 16S rRNA, bla_{KPC}, bla_{OXA-48}, bla_{NDM}, bla_{IMP}, bla_{VIM}, *qnr*A, *qnr*B and *qnr*S genes in the eDNA of the different sampling points of WWTP A (a) and WWTP B (b). Values are expressed in gene copy numbers per milliliter and correspond to the mean ± standard deviation of biological and technical triplicates.

2.4 DISCUSSION

The most abundant phyla and genera found in the two wastewater influents under study are in agreement with previous studies pointing out that the phylogenetic groups

present in the influent samples are generally the same worldwide, despite the differences in their relative abundances that are observed between WWTPs (McLellan et al., 2010; Narciso-da-Rocha et al., 2018; Numberger et al., 2019). Also, the presence of bacteria belonging to several genera associated with potential opportunistic pathogens, namely the genera *Acinetobacter*, *Moraxella* and *Streptococcus* in WWTP A and the genera *Acinetobacter*, *Arcobacter*, *Moraxella* and *Streptococcus* in WWTP B suggests that these can be the main hosts of the target carbapenem and (fluoro)quinolone resistance genes in both wastewater influents and the main responsible for the entrance of these AB resistance genes in the two WWTPs. Both bacterial communities underwent important changes in their compositions during the wastewater treatment process, being most of them tailored by the biological treatment step. In fact, previous studies have already shown that the majority of the bacteria present in the wastewater influents are not able to persist under the operational and environmental conditions of the biological treatment units, highlighting that their impact on the WWTP core community is almost negligible (Hashimoto et al., 2014; Lee et al., 2015; Saunders et al., 2016). The biological reactors are usually designed to simultaneously promote and control the microbial populations that are relevant for the removal of carbon, nitrogen and phosphorus, offering special growth conditions that are not often found in other environments. Therefore, the dilution of the influent load of anthropogenic bacteria combined with their lesser ability to adapt to such specific environments explains the low impact of the wastewater influents on the WWTP core community and the consequent high removal rate of these bacteria during the biological treatment step. However, the results from both the relative abundance of the 16S rRNA gene and the quantification of the target carbapenem and (fluoro)quinolone resistance genes suggest that a fraction of this influent community, which includes several potential opportunistic pathogens, can either pass successfully throughout the two wastewater treatment processes and / or horizontally transfer genetic traits, such as AB resistance genes, to the well-adapted in-house community.

Furthermore, the results confirm that neither biological treatments studied have the ability to completely prevent some of the in-house biomass to continue downstream on the wastewater treatment process, which is in agreement with the literature (Hashimoto et al., 2014; Tong et al., 2019; Xin-chun et al., 2007). Since most of the bacteria present in the wastewater influents are eliminated during the biological treatment step, these in-house bacteria seem to be, indeed, the main hosts of the target AB resistance genes in the treated effluents. Lastly, it is important to mention that the bacterial community composition of each WWTP evolved differently after the biological treatment step, which can be explained by the distinct design and operational conditions of each WWTP. WWTP A applies a BAF technology, where a biofilm support medium (commonly known as carriers) is submerged in the wastewater, creating a large contact area that firstly selects for bacteria that are able to grow attached to a solid surface by the production of specific molecules of the extracellular polymeric matrix. Afterwards, further bacteria are recruited to form a mature attached biofilm. This system is designed to perform a simultaneous biological treatment of the organic matter followed by a physical filtration, targeting the size of the carriers. WWTP B applies a BNR AS technology, in which the activated sludge is formed by microorganisms agglomerating together in suspended biofilms (known as suspended flocs), also due to the production of an extracellular polymeric substance that in this case is not relevant for the adherence to a surface. When the activated sludge is transferred into the settling tanks, the flocs sediment and the treated wastewater is removed from the top. Therefore, and as previously described, each WWTP exerts a distinct selective pressure that shapes differently the corresponding bacterial community (Tong et al., 2019). Altogether, these results reinforce that, regardless of the differences in the design and operational conditions of each WWTP, the conventionally applied wastewater treatments are inefficient in the complete removal of bacteria that can be potentially pathogenic and / or harbour AB resistance

genes from both discharged and reused effluents, contributing to their dissemination into the environment and / or to the human populations.

The high concentrations of carbapenem resistance genes present in the DNA extracted from the bacterial community cells of both wastewater influents reflect the increasing resistance in the community towards this group of last-line ABs. Moreover, their presence is coincident with high relative abundances of several bacteria belonging to genera associated with known carbapenem resistant bacteria, such as the *Acinetobacter* genus, which further reinforces that they can be the main hosts of these genes in the influent samples. Regardless of the treatments applied, the bla_{KPC}, bla_{VIM}, *qnr*B and *qnr*S genes were detected along all sampling points of the two WWTPs. Both biological treatments led to decreases of only 1-2 logs in the concentrations of these AB resistance genes, with no considerable differences in terms of efficiency between them. Since WWTP B has a lower initial load of carbapenem and (fluoro)quinolone resistance genes and also applies a sand filtration step immediately after the biological treatment step, the discharged effluent of this WWTP present lower concentrations of the target AB resistance genes than the discharged effluent of WWTP A. However, these differences are less visible when comparing the reused effluents of both WWTPs, which shows that both cartridge and sand filtrations have similar performances. Also, the addition of sodium hypochlorite to the reused effluents had almost no impact on the concentrations of the target carbapenem and (fluoro)quinolone resistance genes in the two WWTPs, stressing that this disinfection method must be replaced. It is interesting to note that although the bla_{KPC}, bla_{VIM}, *qnr*B and *qnr*S genes still maintain high concentrations in the discharged and reused effluents of both WWTPs, the composition of the bacterial communities is considerably different after the two biological treatment steps. These results are in agreement with the previously described colocalization of both carbapenem and (fluoro)quinolone resistance genes in the same conjugative plasmids (Schultsz and Geerlings, 2012; Strahilevitz et al., 2009) and suggest that the conjugation of such plasmids occurs between the incoming and

the in-house bacteria that find suitable conditions on the biological tanks to efficiently interact and share their mobile genetic elements. Recent studies have already reported the presence of carbapenem and (fluoro)quinolone resistance genes in the wastewater treatment processes of several European WWTPs (Auguet et al., 2017; Cacace et al., 2019; Pärnänen et al., 2019), indicating that the majority of these genes is present at higher concentrations in the Southern European WWTPs rather than in those of Northern Europe (Pärnänen et al., 2019). This is in line with the high concentrations of carbapenem and (fluoro)quinolone resistance genes found in this study. These observations can be explained not only by the differences at the level of the AB consumption pattern between Northern and Southern European countries, but also possibly by the warmer waters of Southern Europe, which may be another favorable factor for the bacterial growth and a major driver of AB resistance in the environment (Pärnänen et al., 2019). Altogether, the results obtained with the DNA extracted from the bacterial community cells point out for the inefficiency of the conventionally applied wastewater treatments in the removal of different carbapenem and (fluoro)quinolone resistance genes from both discharged and reused effluents. This raises the awareness that the legislation on the recycled wastewater quality must be revised taking into consideration the current scientific studies and that the microbial indicators referred are outdated, leaving behind human health menaces, such as AB resistant bacteria and genes. Either transported by their initial bacterial hosts, able to survive to the wastewater treatment process, or horizontally transferred to the well adapted environmental bacteria, the presence of these genes in the discharged and reused effluents make them a source of AB resistance dissemination into the environment and back to the human populations.

Although a few recent studies have started to search for AB resistance genes in the eDNA fraction of different environmental samples (Dong et al., 2019; Guo et al., 2018; Yuan et al., 2019), this is the first study, to the best of our knowledge, to undertake a comprehensive analysis on the concentrations of carbapenem and

(fluoro)quinolone resistance genes in the eDNA fraction of wastewaters. In fact, considerable concentrations of these resistance genes were detected in the eDNA samples of both wastewater influents and, for certain genes, along all sampling points of the two WWTPs. The results support recent research highlighting that the study of the AB resistance in the eDNA should not be neglected, as it can harbour an important fraction of the environmental resistome (Dong et al., 2019; Guo et al., 2018). The bla_{VIM}, *qnr*B and *qnr*S genes were detected along all sampling points of WWTP A, with no important changes in their concentrations detected between the wastewater influent and the treated effluents. In fact, the presence of these three resistance genes in the eDNA fraction, which only represent a minimum percentage of their total concentrations in the influent samples, correspond to, respectively, 23.33 %, 3.50 % and 1.84 % of their total concentrations in the reused effluent. In WWTP B, only the bla_{VIM} gene was detected along all sampling points, being the other four resistance genes found in the corresponding wastewater influent efficiently eliminated during the biological treatment or the sand filtration steps. The eDNA present in these wastewater samples can derive from both cellular extrusion mechanisms or cell lysis, resulting in the release of chromosomal DNA, mobile genetic elements and phages that can be protected against nucleases and able to be assimilated via natural transformation by staying integrated in the extracellular polymeric matrix surrounding the bacteria or adsorbed to colloids, sand particles, clay minerals and humic substances (Dong et al., 2019; Ibáñez de Aldecoa et al., 2017; Pietramellara et al., 2009; Vlassov et al., 2007). Comparing both wastewater treatments, the results clearly show that the BNR AS technology (possibly by retention of the eDNA) and the sand filtration (possibly by eDNA sorption) have a higher efficiency in the removal of the eDNA than the BAF technology and the cartridge filtration. Also, and similar to what was observed in the study of the DNA extracted from the bacterial community cells, the addition of sodium hypochlorite to the reused effluents had no visible impact on the concentrations of the target carbapenem and (fluoro)quinolone resistance genes present in the eDNA

samples of the two WWTPs, once again reinforcing the need for alternative disinfection methods. Altogether, these results demonstrate that the elimination of the eDNA is another important issue that WWTPs will have to overcome when the industry will start to actively tackle the AB related pollution, since it constitutes an additional source for the dissemination of the AB resistance.

2.5 CONCLUSIONS

In this study, it was clearly demonstrated that the WWTPs represent a major vehicle for the dissemination of the AB resistance and was further detailed the great importance of the environmental sector on the AB resistance spreading cycle, underlying the One Health approach. It was possible to conclude that: (1) The biological treatment is the most important step on shaping the bacterial community composition, as well as on affecting the concentrations of the target carbapenem and (fluoro)quinolone resistance genes along the wastewater treatment process; (2) Regardless of the different designs and treatments applied in the two WWTPs, carbapenem and (fluoro)quinolone resistance genes persisted at high concentrations in both DNA and eDNA fractions of the discharged and reused effluents; (3) The conventionally applied treatments for wastewater reuse are clearly inefficient, being this stream a direct gateway for the dissemination of AB resistant bacteria and genes back to the human populations; (4) The eDNA fraction of the wastewater is a relevant source of carbapenem and (fluoro)quinolone resistance genes, representing an additional path for the dissemination of the AB resistance. For wastewater reuse to become a safe and reliable practice in the near future, able to be implemented as a sustainable alternative in areas such as agricultural irrigation, targeted treatments towards AB resistant bacteria and genes must be developed and / or implemented at full-scale in the WWTPs.

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CHAPTER 3

Environmental and pathogenic carbapenem resistant bacteria isolated from a wastewater treatment plant harbour distinct antibiotic resistance mechanisms

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ABSTRACT

Wastewater treatment plants are important reservoirs and sources for the dissemination of antibiotic resistance into the environment. Here, two different groups of carbapenem resistant bacteria – the potentially environmental and the potentially pathogenic – were isolated from both the wastewater influent and discharged effluent of a full-scale wastewater treatment plant and characterized by whole genome sequencing and antibiotic susceptibility testing. Among the potentially environmental isolates, there was no detection of any acquired antibiotic resistance genes, which supports the idea that their resistance mechanisms are mainly intrinsic. On the contrary, the potentially pathogenic isolates presented a broad diversity of acquired antibiotic resistance genes towards different antibiotic classes, especially β-lactams, aminoglycosides and (fluoro)quinolones. All these bacteria showed multiple β-lactamase-encoding genes, some with carbapenemase activity, such as the bla_{KPC}-type genes found in the *Enterobacteriaceae* isolates. The antibiotic susceptibility testing assays performed on these isolates also revealed that all had a multiresistance phenotype, which indicates that the acquired resistance is their major antibiotic resistance mechanism. In conclusion, the two bacterial groups have distinct resistance mechanisms, which suggest that the antibiotic resistance in the environment can be a more complex problematic than what is generally assumed.

Keywords: Antibiotic resistance; Carbapenems; Wastewater treatment plants; Discharged effluents; Environmental and pathogenic carbapenem resistant bacteria

3.1 INTRODUCTION

The increasing dissemination of carbapenem resistant bacteria represents a major worldwide problem and an important threat to human health (Nordmann and Poirel, 2019). Despite this being a concern generally associated with health care facilities, the presence of carbapenem resistant bacteria is also increasing in wastewater treatment plants (WWTPs), which are already considered relevant anthropogenic reservoirs and sources for the spread of the antibiotic (AB) resistance into the environment (Guo et al., 2017; Michael et al., 2013; Rizzo et al., 2013).

Carbapenem resistance can result from both enzyme-mediated and / or non-enzyme mediated processes of bacteria (Nordmann et al., 2012; Nordmann and Poirel, 2019). The enzyme-mediated resistance mechanisms are encoded by specific *bla* genes and involve the hydrolysis of these ABs by carbapenemases, a particular group of β-lactamases that hydrolyze not only carbapenems but also other important β-lactam ABs, such as penicillins, cephalosporins and monobactams (Nordmann et al., 2012; Nordmann and Poirel, 2019; Poirel et al., 2007; Walsh, 2010). In terms of clinical relevance and global distribution, the most important carbapenemases are: (1) Class A serine-β-lactamases, encoded by bla_{KPC}-type genes; (2) Class B metallo-β-lactamases, encoded by bla_{NDM}, bla_{IMP} and bla_{VIM}-type genes; and (3) Class D serine-β-lactamases, encoded by $bla_{OXA\text{-}48}$-type genes (Nordmann et al., 2012; Pfeifer et al., 2010). These carbapenemase-encoding genes can be found in the bacterial chromosome, but more often in conjugative plasmids, which promotes their horizontal transfer between resistant and non-resistant bacteria. In fact, the plasmid transfer and acquisition is the main driver of the rapid increase and global spread of the carbapenem resistance that has been observed in the last decade (Nordmann et al., 2012; Nordmann and Poirel, 2019; Pfeifer et al., 2010; Schultsz and Geerlings, 2012). However, bacteria can also be resistant to carbapenems due to mutations causing loss of expression of porin-encoding genes; as a result of the overexpression of genes encoding for efflux pumps; or due to mutations that modify the production levels or the binding affinities of the

penicillin-binding proteins (Nordmann et al., 2012; Nordmann and Poirel, 2019). These non-enzyme mediated resistance mechanisms, also known as intrinsic resistance mechanisms, can occur alone or together with the production of extended-spectrum β-lactamases, cephalosporinases and / or carbapenemases, generating well-known carbapenem resistance phenotypes (Nordmann et al., 2012; Nordmann and Poirel, 2019).

Several recent studies already point out for the existence of high concentrations of carbapenemase-encoding genes and corresponding carbapenem resistant bacteria along all the main steps of different wastewater treatment processes worldwide, from the wastewater influents to the treated effluents, warning for their subsequent release into the water bodies (Cacace et al., 2019; Lamba and Ahammad, 2017; Mathys et al., 2019; Oliveira et al., 2020; Pärnänen et al., 2019; Yang et al., 2016). Nevertheless, most of these studies have especially focused on the detection and quantification of target genes by PCR / qPCR techniques. This has led to a serious gap in the isolation of the different populations of carbapenem resistant bacteria that exist in the wastewater environments and on the assessment and characterization of their acquired resistance genes and intrinsic resistance mechanisms towards carbapenems and other ABs. The work developed by Hrenovic et al. (Hrenovic et al., 2019a, 2019b, 2017a, 2017b) explores the isolation of different populations of carbapenem resistant bacteria from the wastewater environments using two incubation temperatures in selective culture media: (1) Incubation at 37 °C for the isolation of presumably environmental carbapenem resistant bacteria, whose resistance mechanisms are thought to be mainly intrinsic; (2) Incubation at 42 °C for the isolation of presumably pathogenic carbapenem resistant bacteria, whose resistance mechanisms are thought to be mainly acquired. However, these studies mostly address the physicochemical characterization of the different wastewater environments and the abundance of these populations of carbapenem resistant bacteria along the wastewater treatment processes. Therefore, a deeper genotypical and phenotypical characterization of environmental and

pathogenic isolates is still crucial for a better understanding of these increasingly important AB resistance reservoirs. Accordingly, the aim of the present study was to genotypically and phenotypically characterize the AB resistance profile of several environmental and pathogenic carbapenem resistant bacteria isolated from a full-scale WWTP.

3.2 MATERIALS AND METHODS

3.2.1 WWTP description and sample collection

Samples were collected from a Portuguese full-scale WWTP designed to treat the domestic wastewater of approximately 756,000 population equivalents (P.E.) employing the biological aerated filters technology. Two sampling points were defined and three biological samples of 10 L each were collected from the wastewater influent and discharged effluent in sterile containers in November of 2019. After collection, all samples were directly transported to the laboratory under refrigerated conditions and immediately processed upon arrival.

3.2.2 Determination of carbapenem resistant bacteria concentrations

To determine the concentrations of carbapenem resistant bacteria in the wastewater influent and discharged effluent, ten-fold serial dilutions of each sampling point were made in 1 L sterile saline solution and 100 mL samples of each dilution were filtered in triplicate through sterile 0.22 µm pore-size polyethersulfone (PES) filters (Pall Corporation, New York, NY, USA). Then, the filters were placed on selective and chromogenic CHROMagar™ mSuperCARBA™ plates (CHROMagar, Paris, France) and incubated at 30 °C or 42 °C for 24 h. The different incubation temperatures were adapted from the work developed by Hrenovic et al. (Hrenovic et al., 2019a, 2019b, 2017a, 2017b) and used to distinguish between potentially environmental bacteria, which are normally able to grow at 30 °C, and potentially pathogenic bacteria, which

are usually also able to survive and grow at higher temperatures. Since many environmental bacteria have optimal growth temperatures below 37 °C, their isolation was performed at 30 °C instead of 37 °C, so that as few different species as possible would have their growth inhibited due to the temperature used. Following incubation, the colonies obtained on each plate were enumerated and their concentrations were calculated and expressed as colony-forming units per milliliter (CFU / mL).

3.2.3 Isolation, DNA extraction and species identification of carbapenem resistant bacteria

A subset of colonies of each incubated plate showing different phenotypes were randomly picked and sub-cultured in the same medium and conditions until pure colonies were obtained. The pure colonies were inoculated in Tryptic Soy Broth (TSB) (VWR, Radnor, PA, USA) supplemented with 0.5 µg / mL meropenem trihydrate (Sigma-Aldrich, Saint Louis, MO, USA) and again incubated at 30 °C or 42 °C (depending on its original growth temperature) with shaking (150 rpm), overnight. After incubation, the bacterial DNA was extracted using the standard protocol from the DNeasy® UltraClean® Microbial Kit (Qiagen, Hilden, Germany) and the DNA concentrations and purities were measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The extracted DNAs were then used for the species identification, which was performed by 16S rRNA gene sequencing. Briefly, the 16S rRNA genes from each colony were amplified by PCR with the primer pair Fw 5'-GCCAGCAGCCGCGGTAA-3' and Rv 5'-AAGGAGGTGATCCRGCCGCA-3' (adapted from (Turner et al., 1999)) in a mix reaction containing 12.5 µL NZYTaq II 2x Green MasterMix (NZYTech, Lisbon, Portugal), 200 nM of each forward and reverse primers, 50 ng of DNA template and nuclease free water (to complete 25 µL). The PCR reactions were conducted in a Doppio Termocycler (VWR, Radnor, PA, USA) using the following program: Initial denaturation at 95 °C for 3 min; 35 cycles of amplification at 94 °C for 30 s, 57 °C for 30 s and 72 °C for 45 s; final elongation at 72 °C for 5 min. The PCR products were then sent to Eurofins

Genomics (Ebersberg, Germany) for purification and Sanger Sequencing and the resulting sequences were aligned against the National Centre for Biotechnology (NCBI) 16S rRNA gene database using the BLASTn algorithm to determine the taxonomic identities.

3.2.4 Screening of carbapenem resistance genes

The extracted DNAs were also used to perform a screening of five important carbapenem resistance genes in terms of clinical relevance and global distribution – *bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{NDM}$, *bla*$_{IMP}$ and *bla*$_{VIM}$ – by two previously developed *TaqMan* multiplex qPCRs (Oliveira et al., 2020). The *TaqMan* multiplex qPCR 1 was designed for the detection and quantification of *bla*$_{KPC}$ and *bla*$_{OXA-48}$-type genes, whereas the *TaqMan* multiplex qPCR 2 was designed for the detection and quantification of *bla*$_{NDM}$, *bla*$_{IMP}$ and *bla*$_{VIM}$-type genes. The *TaqMan* multiplex qPCR assays were conducted in triplicate on a LightCycler 96 Real-Time PCR System (Roche, Basel, Switzerland) using the following program: DNA denaturation / polymerase activation at 95 ° for 5 min; 40 cycles of amplification at 95 °C for 10 s and 60 °C for 30 s. Information about the primers and probes is provided in Table S3.1 and the composition of the mix reactions is provided in Table S3.2.

3.2.5 Whole genome sequencing and assembly

Based on the species identification and on the screening of carbapenem resistance genes, the isolate with the biggest number and diversity of the target carbapenem resistance genes from each different species isolated from the discharged effluent samples at 30 °C and 42 °C was chosen to perform an enhanced whole genome sequencing service at MicrobesNG (Birmingham, UK), which combines two distinct technologies: The Illumina short reads and the Oxford Nanopore long reads.

For the Illumina sequencing, plated cultures of each bacterial isolate were inoculated into a cryoperservative (Microbank, Pro-Lab Diagnostics, Richmond Hill, ON, Canada). Then, between 10 and 20 microliters of each suspension were lysed with 120 µL of Tris-EDTA (TE) buffer containing lysozyme (in a final concentration of 0.1 mg / mL) and RNase A (ITW Reagents, Barcelona, Spain) (in a final concentration of 0.1 mg / mL) and incubated at 37 °C for 25 min. After that, proteinase K (VWR, Radnor, USA) (in a final concentration of 0.1 mg / mL) and SDS (Sigma-Aldrich, Saint Louis, MO, USA) (in a final concentration of 0.5 % v / v) were added and the mixture was incubated at 65 °C for 5 min. The genomic DNA was then purified using an equal volume of solid phase reversible immobilization beads, resuspended in EB buffer (Qiagen, Hilden, Germany) and quantified with the Quant-iT dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) in an Eppendorf AF2200 plate reader (Eppendorf, Hamburg, Germany). The genomic DNA libraries were prepared on a Hamilton Microlab STAR automated liquid handling system (Hamilton Bonaduz AG, Rapperswil-Jona, Switzerland) using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions, with the following modifications: (1) 2 ng of DNA were used as input; (2) PCR elongation time was increased from 30 s to 1 min. The pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina (Roche, Basel, Switzerland) on a LightCycler 96 Real-Time PCR System (Roche, Basel, Switzerland) and then sequenced with the Illumina HiSeq (Illumina, San Diego, CA, USA) using a 250 bp paired end protocol.

For the Oxford Nanopore sequencing, broth cultures of each bacterial isolate were pelleted out and then resuspended in a cryoperservative (Microbank, Pro-Lab Diagnostics, ON, Canada). Then, approximately 2 x 10^9 cells were used for high molecular weight DNA extraction with the Nanobind CCB Big DNA Kit (Circulomics, Baltimore, MD, USA). The DNA was quantified with the Qubit dsDNA HS Assay (Thermo Fisher Scientific, Waltham, MA, USA) in a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and the long read genomic DNA libraries were then

prepared with both the Oxford Nanopore SQK-RBK004 Kit and / or with the SQK-LSK109 Kit with Native Barcoding EXP-NBD104/114 (Oxford Nanopore Technologies, Oxford, UK) using 400-500 ng of high molecular weight DNA. At the end, 12 to 24 barcoded samples were pooled together into a single sequencing library and loaded in a FLO-MIN106 (R.9.4 or R.9.4.1) flow cell in a GridION (Oxford Nanopore Technologies, Oxford, UK).

The Illumina reads were trimmed using Trimmomatic v.0.30 (Bolger et al., 2014) with a sliding window quality cut-off of Q15, the genome assemblies were performed with Unicycler v.0.4.0 (Wick et al., 2017) and the contigs were annotated using Prokka v.1.11 (Seemann, 2014).

3.2.6 Taxonomic confirmation and identification of acquired AB resistance genes, conjugative plasmids and virulence factors

The taxonomic confirmation was conducted using the tools SpeciesFinder 2.0 and KmerFinder 3.2 (Larsen et al., 2014). Then, the identification of acquired AB resistance genes was performed using ResFinder 4.0 (Bortolaia et al., 2020), with a threshold of 90 % identity and a minimum length of 60 %, and KmerResistance 2.2 (Clausen et al., 2018, 2016), with thresholds of 70 % identity and 10 % depth corr. The detection of conjugative plasmids in the *Enterobacteriaceae* genomes was conducted using PlasmidFinder 2.0 (Carattoli et al., 2014) and the presence of virulence factors in the *Escherichia coli* isolates was assessed using VirulenceFinder 2.0 (Joensen et al., 2014), with a threshold of 90 % identity and a minimum length of 60 %.

3.2.7 AB susceptibility testing

The AB resistance phenotypes of the potentially pathogenic carbapenem resistant bacteria isolated from the discharged effluent samples were determined according to the EUCAST disk diffusion method on Mueller-Hinton agar (BD Difco, Franklin Lakes, NJ,

USA) (EUCAST, 2020). From the ABs tested, four belong to the β-lactam class – ampicillin 10 μg (AMP10), cefotaxime 5 μg (CTX5), imipenem 10 μg (IMP10) and meropenem 10 μg (MEM10) (Oxoid, Ottawa, ON, Canada) – and six belong to AB classes for which different acquired resistance genes were found in the whole genomes of some bacterial isolates – chloramphenicol 30 μg (C30), ciprofloxacin 5 μg (CIP5), gentamicin 10 μg (CN10), tetracycline 30 μg (TE30), trimethoprim 5 μg (W5) and trimethoprim + sulphamethoxazole 25 μg (SX25) (Oxoid, Ottawa, Canada). These ABs were chosen due to their wide use in the treatment of different bacterial infections. Triplicates of each bacterial isolate were adjusted to the 0.5 McFarland standard concentration and inoculated on Mueller-Hinton agar plates. Then, the antimicrobial disks were applied and the plates were incubated at 35 ± 1 °C for 18 ± 2 h. After incubation, the inhibition zone diameters were measured and the bacterial isolates were categorized as "S" (susceptible, standard dosing regimen), "I" (susceptible, increased exposure), or "R" (resistant) (Kahlmeter and The EUCAST Steering Committee, 2019). Bacterial isolates presenting a resistance phenotype to, at least, three different AB classes were considered multiresistant. The strain *Escherichia coli* ATCC 25922 was used as a control of these AB susceptibility testing assays.

3.3 RESULTS

3.3.1 Concentrations of carbapenem resistant bacteria

Potentially environmental and potentially pathogenic carbapenem resistant bacteria were detected at high concentrations in wastewater influent and discharged effluent samples (Table S3.3). The potentially environmental carbapenem resistant bacteria – isolated after incubation at 30 °C – presented concentrations of 9.58 x 10^4 CFU / mL in the wastewater influent and 5.37 x 10^3 CFU / mL in the discharged effluent, whereas the potentially pathogenic carbapenem resistant bacteria – isolated after incubation at 42 °C – presented concentrations of 1.38 x 10^3 CFU / mL in the wastewater

influent and 1.20 x 10^2 CFU / mL in the discharged effluent (Table S3.3). All bacterial concentrations correspond to the mean of the mean values obtained for the technical triplicates of each of the three biological samples collected for each sampling point.

3.3.2 Species identification by 16S rRNA gene sequencing and screening of carbapenem resistance genes

Thirty-two carbapenem resistant isolates were obtained from wastewater influent samples incubated at 30 °C. The taxa identified were: *Pseudomonas* spp. (n = 16), namely the species *P. entomophila* (1), *P. fluorescens* (3), *P. fragi* (1), *P. lundensis* (1), *P. migulae* (1), *P. psychrophila* (1), *P. putida* (7) and *P. syringae* (1); *Aeromonas* spp. (n = 15), namely the species *A. caviae* (7), *A. salmonicida* (1) and *A. veronii* (7); and *Raoultella ornithinolytica* (n = 1) (Table S3.4). The screening of carbapenem resistance genes revealed the presence of bla_{KPC}-type genes in eight of these bacterial isolates (Table S3.4). Twenty-six carbapenem resistant isolates were obtained from the discharged effluent samples incubated at 30 °C. The taxa identified were: *Pseudomonas* spp. (n = 13), namely the species *P. entomophila* (1), *P. fluorescens* (2), *P. fragi* (2), *P. monteilii* (1), *P. psychrophila* (1) and *P. putida* (6); *Aeromonas* spp. (n = 9), namely the species *A. caviae* (1) and *A. veronii* (8); *Chromobacterium rhizoryzae* (n = 3); and *Acinetobacter pittii* (n = 1) (Table S3.4). In the screening of carbapenem resistance genes, there was no detection of any of the five target resistance genes in those bacterial isolates (Table S3.4).

For the incubation at 42 °C, twenty-seven carbapenem resistant isolates were obtained from the wastewater influent samples. The taxa identified were: *Acinetobacter* spp. (n = 11), namely the species *A. baumannii* (6) and *A. pittii* (5); *Escherichia coli* (n = 6); *Citrobacter* spp. (n = 3), namely the species *C. amalonaticus* (1) and *C. freundii* (2); *Klebsiella* spp. (n = 3), namely the species *K. pasteurii* (1) and *K. pneumoniae* (2); *Enterobacter asburiae* (n = 2); and *Raoultella ornithinolytica* (n = 2) (Table S3.5). The screening of carbapenem resistance genes revealed the presence of

bla$_{KPC}$-type genes in nineteen bacterial isolates, *bla*$_{OXA-48}$-type genes in two bacterial isolates and *bla*$_{VIM}$-type genes in seven bacterial isolates (Table S3.5). Twenty-two carbapenem resistant isolates were obtained from the discharged effluent samples. The taxa identified were: *Klebsiella pneumoniae* (n = 8); *Acinetobacter* spp. (n = 6), namely the species *A. baumannii* (5) and *A. pittii* (1); *Escherichia coli* (n = 4); *Aeromonas veronii* (n = 2); and *Citrobacter* spp. (n = 2), namely the species *C. amalonaticus* (1) and *C. freundii* (1) (Table S3.5). The screening of carbapenem resistance genes revealed the presence of *bla*$_{KPC}$-type genes in fourteen bacterial isolates and *bla*$_{VIM}$-type genes in one bacterial isolate (Table S3.5).

After both the species identification and the screening of carbapenem resistance genes in the bacterial isolates obtained from the discharged effluent samples, ten of the bacterial isolates grown at 30 °C and seven of bacterial isolates grown at 42 °C (one bacterial isolate from each of the species identified for both incubation temperatures) were selected to perform a whole genome sequencing analysis.

3.3.3 Taxonomic confirmation using the whole genome sequencing data

Both the SpeciesFinder 2.0 and the KmerFinder 3.2 tools positively confirmed the genus of the ten bacterial isolates grown at 30 °C (Table S3.6). Regarding the species level, the SpeciesFinder 2.0 tool confirmed the identification of three of these isolates and the KmerFinder 3.2 tool confirmed the identification of seven of these isolates (Table S3.6). For the identification of the bacterial isolates grown at 42 °C, the SpeciesFinder 2.0 tool positively confirmed the genus of six bacterial isolates and the species of four of these isolates, whereas the KmerFinder 3.2 tool positively confirmed the genus of the seven bacterial isolates and the species of five of these isolates (Table S3.7). The taxonomic identifications obtained with the KmerFinder 3.2 tool will be used from now on in this article, since this is considered to be the most accurate tool for the identification of different bacterial strains (Larsen et al., 2014). Therefore, the *Pseudomonas psychrophila*, *Pseudomonas putida* and *Acinetobacter pittii* isolates –

grown at 30 °C – will now be renamed as *Pseudomonas fragi*, *Pseudomonas* sp. URMO17WK12:I11 and *Acinetobacter oleivorans*, respectively, and the *Citrobacter amalonaticus* and *Citrobacter freundii* isolates – grown at 42 °C – will now be renamed as *Citrobacter* sp. Y3 and *Citrobacter portucalensis*, respectively.

3.3.4 Identification of acquired AB resistance genes, conjugative plasmids and virulence factors

3.3.4.1 Acquired AB resistance genes

After the analysis using both the ResFinder 4.0 and KmerResistance 2.2 tools, three bacterial isolates grown at 30 °C, namely the *A. oleivorans*, *A. caviae* and *A. veronii* isolates presented different acquired AB resistance genes, including acquired β-lactam resistance genes, in their whole genomes (Figure 3.1; Table S3.8). Besides the presence of β-lactam resistance genes, aminoglycoside, (fluoro)quinolone, macrolide, phenicol, rifampicin, sulphonamide and trimethoprim resistance genes were also detected. However, in the remaining seven bacterial isolates grown at 30 °C – *C. rhizoryzae*, *P. entomophila*, *P. fluorescens*, *P. fragi* (2), *P. monteilii* and *Pseudomonas* sp. URMO17WK12:I11 – there was no detection of any acquired AB resistance genes (Figure 3.1; Table S3.8).

Regarding the bacterial isolates grown at 42 °C, the analysis performed with the ResFinder 4.0 and KmerResistance 2.2 tools revealed that all bacterial isolates, namely the *A. baumannii*, *A. pittii*, *A. veronii*, *C. portucalensis*, *Citrobacter* sp. Y3, *E. coli* and *K. pneumoniae* isolates, presented different acquired AB resistance genes, including acquired β-lactam resistance genes, in their whole genomes (Figure 3.1; Table S3.9). Besides the presence of the β-lactam resistance genes, aminoglycoside, colistin, (fluoro)quinolone, fosfomycin, macrolide, phenicol, sulphonamide, tetracycline and trimethoprim resistance genes were also abundantly detected. In fact, a total of about

71 % of the bacterial isolates grown at 42 °C presented acquired resistance genes to, at least, three different AB classes (Figure 3.1; Table S3.9).

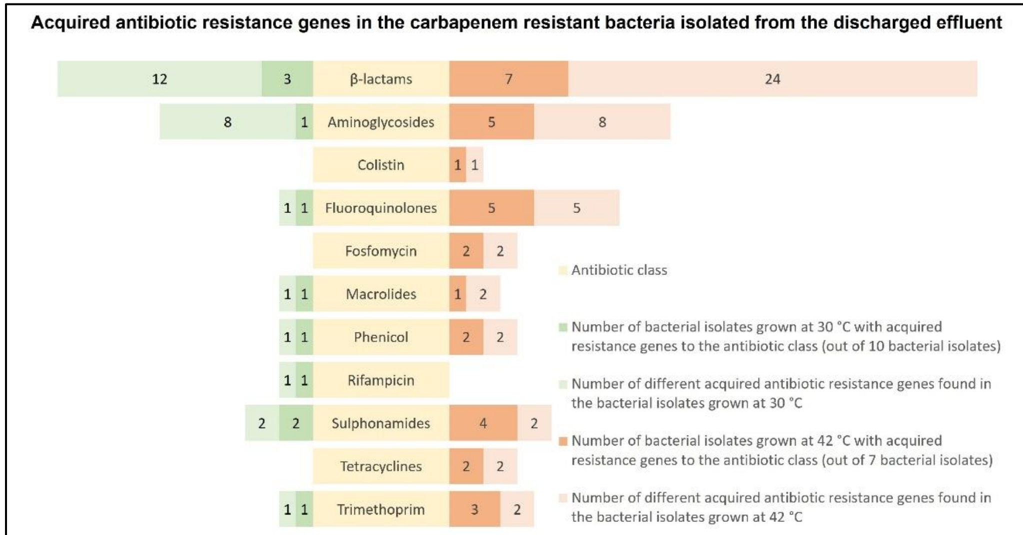

Figure 3.1 | Number of bacterial isolates grown at 30 °C (green) and 42 °C (orange) from the discharged effluent samples harbouring acquired resistance genes towards different AB classes (yellow) and corresponding diversity of acquired AB resistance genes (light green and light orange).

3.3.4.2 Conjugative plasmids in *Enterobacteriaceae* spp. and virulence factors in *E. coli*

According to the PlasmidFinder 2.0 tool, which identifies plasmids in total or partial sequenced isolates of *Enterobacteriaceae* spp., all carbapenem resistant *Enterobacteriaceae* isolated from the discharged effluent samples – *C. portucalensis*, *Citrobacter* sp. Y3, *E. coli* and *K. pneumoniae* – harbour different plasmids known to contain the previously described acquired AB resistance genes (Figure 3.2). Moreover, the VirulenceFinder 2.0 tool, which identifies virulence genes in sequenced *E. coli* isolates, revealed the presence of the *ast*A, *gad*, *iss*, *lpf*A, *omp*T, *sit*A, *ter*C and *tra*T virulence factors in the whole genome of the *E. coli* isolate (Figure 3.2).

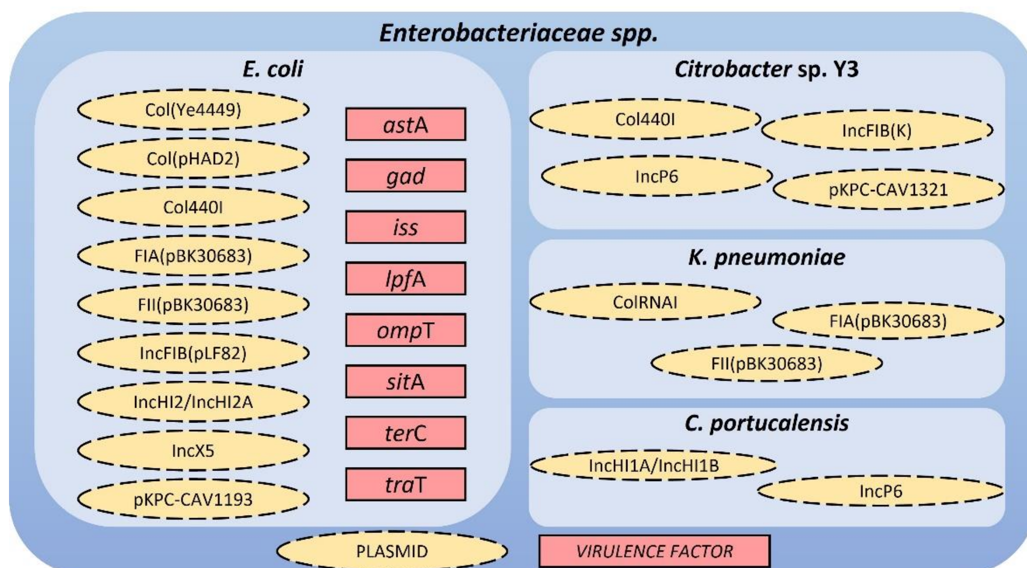

Figure 3.2 | Plasmids found in the whole genomes of the carbapenem resistant *Enterobacteriaceae* isolated from the discharged effluent samples and virulence factors found in the *E. coli* isolate.

3.3.5 AB susceptibility testing

Apart from the *A. caviae* and *A. veronii* (2) isolates, for which there are no EUCAST breakpoints available for most of the ABs used, all bacterial isolates, namely *A. baumannii*, *A. oleivorans*, *A. pittii*, *C. portucalensis*, *Citrobacter* sp. Y3, *E. coli* and *K. pneumoniae*, showed resistance phenotypes towards ampicillin and cefotaxime and, apart from the *A. oleivorans* and *A. pittii*, to imipenem and meropenem (Table 3.1). Besides the resistance phenotypes to the β-lactam class, all bacterial isolates presented resistance phenotypes to (fluoro)quinolones (ciprofloxacin) and tetracyclines (tetracycline) (Table 3.1). Moreover, most bacterial isolates also showed resistance phenotypes towards chloramphenicol, gentamicin, trimethoprim and trimethoprim + sulphamethoxazole (Table 3.1). All bacterial isolates showed resistance phenotypes to more than three AB classes, being considered multiresistant.

Table 3.1 | AB resistance phenotype of the potentially pathogenic carbapenem resistant bacteria isolated from the discharged effluent samples.

Bacteria	AMP10	CTX5	IPM10	MEM10	CIP5	C30	CN10	TE30	W5	SXT25
Acinetobacter baumannii	R	R	R	R	R	R	R	R	R	S
Acinetobacter pittii	R	R	I	I	R	R	R	R	R	S
Acinetobacter oleivorans	R	R	I	I	R	R	S	R	R	R
Aeromonas caviae	*	*	*	*	S	*	*	*	*	R
Aeromonas veronii (1)	*	*	*	*	R	*	*	*	*	R
Aeromonas veronii (2)	*	*	*	*	R	*	*	*	*	S
Citrobacter portucalensis	R	R	R	R	R	R	R	R	R	R
Citrobacter sp. Y3	R	R	R	R	R	R	R	R	S	S
Escherichia coli	R	R	R	R	R	S	R	R	R	R
Klebsiella pneumoniae	R	R	R	R	R	S	R	R	R	R

AMP10 – Ampicillin 10 µg; CTX5 – Cefotaxime 5 µg; IMP10 – Imipenem 10 µg; MEM10 – Meropenem 10 µg; C30 – Chloramphenicol 30 µg; CIP5 – Ciprofloxacin 5 µg; CN10 – Gentamicin 10 µg; TE30 – Tetracycline 30 µg; W5 – Trimethoprim 5 µg; SXT25 – Trimethoprim + sulphamethoxazole 25 µg.

* No EUCAST breakpoints available; S – Susceptible, standard dosing regimen; I – Susceptible, increased exposure; R – Resistant.

3.4 DISCUSSION

The presence of high concentrations of carbapenem resistant bacteria in both the wastewater influent and discharged effluent samples is not only a reflection of the increasing use of carbapenems in recent years, but also an additional evidence regarding the inefficiency of the conventionally applied wastewater treatments in the elimination of these microorganisms from the treated effluents, subsequently leading to their release into the environment (Oliveira et al., 2020).

The incubation of the wastewater influent and discharged effluent samples in selective media at 30 °C and 42 °C allowed the isolation and characterization of two different populations of carbapenem resistant bacteria: The potentially environmental

and the potentially pathogenic (Hrenovic et al., 2019a, 2019b, 2017a, 2017b). At 30 °C, it is expected that both grow. However, and given the environmental nature of the samples, the proportion of environmental bacteria will be much higher than the proportion of pathogenic bacteria. Thus, when randomly picking colonies, the odds of picking an environmental carbapenem resistant bacteria are much higher than the odds of picking a pathogenic carbapenem resistant bacteria. Concordantly, and despite the possible bias induced by this methodology, in which pathogenic carbapenem resistant bacteria are also able to grow in the environmental carbapenem resistant bacteria plates, at 30 °C, most of the identified bacteria were described as having an environmental origin, although three potentially pathogenic bacteria for both humans and animals were also isolated. In general, these environmental carbapenem resistant bacteria presented very low detection rates of *bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{NDM}$, *bla*$_{IMP}$ and *bla*$_{VIM}$-type genes, raising the hypothesis that most of them could be intrinsically resistant to these ABs. On the contrary, most of the identified bacteria at 42 °C were well-known human and animal pathogens. These bacteria presented much higher detection rates of the target carbapenem resistance genes, suggesting that they are resistant to carbapenems mainly as a result of the expression of these and other acquired resistance genes.

To have a deeper knowledge on the distinct resistance mechanisms of both the potentially environmental and potentially pathogenic carbapenem resistant bacteria obtained from the discharged effluent samples, the genotype of a bacterial isolate from each species obtained at 30 °C and 42 °C was characterized through a whole genome sequencing analysis, which, in a first approach, allowed the taxonomic confirmation of all bacteria. The whole genome sequencing data revealed that the potentially environmental carbapenem resistant bacteria did not harbour any acquired AB resistance genes, which corroborates the previous *TaqMan* multiplex qPCR results and supports the idea that their resistance to carbapenems may result from intrinsic mechanisms. This could be explained by the occurrence of mutations causing loss of

expression of porin-encoding genes, the overexpression of genes encoding for efflux pumps, or the occurrence of mutations that modify the production levels or the binding affinities of the penicillin-binding proteins (Nordmann et al., 2012; Nordmann and Poirel, 2019). In fact, studies are starting to report that the majority of the carbapenem resistant bacteria of environmental origin present in aquatic environments, namely *Chromobacterium* spp. and *Pseudomonas* spp. isolates, have different intrinsic mechanisms of AB resistance (Hrenovic et al., 2019a, 2019b, 2017a, 2017b; Mathys et al., 2019; Tacão et al., 2015). Generally, these bacteria are not considered of epidemiological relevance, since most of them are very unlikely to generate human or animal infections and to horizontally transfer AB resistance genes to other bacteria (Mathys et al., 2019). For the potentially pathogenic carbapenem resistant bacteria isolated from the discharged effluent samples (three isolated at 30 °C – it happened despite the odds favoring the pick of environmental carbapenem resistant bacteria – and seven isolated at 42 °C), the whole genome sequencing data revealed the presence of a vast diversity of acquired AB resistance genes. Most of these bacteria represent important human and animal pathogens, able to cause a variety of nosocomial infections with frequent negative patient outcomes. In fact, the World Health Organization has already classified the carbapenem resistant *A. baumannii*, *E. coli* and *K. pneumoniae* as microorganisms of critical priority for the research, discovery and development of new ABs, due to their increasing spread worldwide and to the urgent need of new effective treatments (WHO, 2017). These ten bacterial isolates presented multiple acquired β-lactamase-encoding genes, some of which with carbapenemase activity. Among the acquired carbapenemase-encoding genes, *bla*$_{KPC}$-type genes were found in the four *Enterobacteriaceae* isolates (*C. portucalensis*, *Citrobacter* sp. Y3, *E. coli* and *K. pneumoniae*), which agrees with previous studies reporting the predominance of these genes in different clinical isolates of carbapenemase-producing *Enterobacteriaceae* already responsible for serious outbreaks in Portugal (Aires-de-sousa et al., 2019; Manageiro et al., 2018, 2015; Vubil et al., 2017). Concordantly, all

four *Enterobacteriaceae* isolates also carry IncF or IncP-like plasmids, well known for their major roles in the dissemination not only of bla_{KPC}-type genes, but also of other AB resistance genes among *Enterobacteriaceae* (Manageiro et al., 2018, 2015; Yao et al., 2017). Besides the β-lactam resistance genes, acquired resistance genes towards other important AB classes, such as aminoglycosides and (fluoro)quinolones, were also present with high detection rates in the potentially pathogenic carbapenem resistant isolates, which was expected due to the common co-occurrence of β-lactam, aminoglycoside and (fluoro)quinolone resistance genes in the same conjugative plasmids (Schultsz and Geerlings, 2012; Zhang et al., 2020). Moreover, among the acquired AB resistance genes towards other AB classes found in some bacterial isolates, the most concerning situation was the detection of the newly identified *mcr*-9 gene variant, conferring resistance towards colistin and frequently present in IncHI2-like plasmids, in the *E. coli* isolate (Li et al., 2020). Since colistin is currently one of the most effective last-resort ABs used in the treatment of severe infections caused by carbapenem resistant bacteria, the rapid development and spread of the mobilized colistin resistance (*mcr*) genes represent another serious public health challenge (Li et al., 2020; Poirel et al., 2017). In fact, the *E. coli* isolate presented not only a great diversity of acquired AB resistance genes and conjugative plasmids, but also ten different virulence factors, having one of the most alarming genetic profiles among the bacterial isolates obtained from the discharged effluent samples. Altogether, the obtained results suggest that the great majority of the potentially pathogenic carbapenem resistant bacteria relies on the acquisition and expression of AB resistance genes as their main resistance mechanism, in opposition to the potentially environmental carbapenem resistant bacteria, which appear to be resistant to different ABs especially through intrinsic mechanisms, alone or in combination.

The analysis of the AB resistance phenotypes of the potentially pathogenic carbapenem resistant bacteria isolated from the discharged effluent samples revealed that all were multiresistant, showing resistance phenotypes to more than three AB

classes. These results reinforce the importance of the conjugative plasmids in the simultaneous dissemination of different AB resistance genes and is in line with the reported occurrence of multiresistant bacteria in the Portuguese wastewater environments (Amador et al., 2015). Furthermore, only the *A. oleivorans* and *A. pittii* bacterial isolates showed an intermediate susceptibility phenotype towards carbapenems (imipenem and meropenem), only the *A. oleivorans* bacterial isolate presented susceptibility to aminoglycosides (gentamicin) and only the *Citrobacter* sp. Y3 bacterial isolate was susceptible to trimethoprim. These results not only show that the acquired AB resistance genes are expressed and originate the corresponding resistance phenotypes, but also demonstrate that some of these bacteria also have intrinsic resistance mechanisms to ABs for which no acquired AB resistance genes were found in their whole genomes.

3.5 CONCLUSIONS

This study allowed the isolation of two different populations of carbapenem resistant bacteria from the wastewater influent and discharged effluent of a full-scale WWTP – the potentially environmental and the potentially pathogenic – and the genotypical and phenotypical characterization of their AB resistance profile. It was possible to observe that: (1) Although with a reduction in the concentrations of CFU between the wastewater influent and discharged effluent, both potentially environmental and potentially pathogenic fractions of carbapenem resistant bacteria were present at high concentrations in the discharged effluent samples; (2) The potentially environmental carbapenem resistant bacteria presented low detection rates of acquired AB resistance genes, appearing to be resistant to carbapenems and to other ABs mainly through intrinsic mechanisms; (3) The potentially pathogenic carbapenem resistant bacteria presented high detection rates of acquired resistance genes towards carbapenems and other important AB classes, and their major AB resistance mechanism appears to be their acquisition and expression. Altogether, and

underlying the One Health approach, the obtained results prove that the conventionally applied wastewater treatments are inefficient in the elimination of these microorganisms from the discharged effluents. These streams thus act as important vehicles for the dissemination of potentially pathogenic AB resistant bacteria and corresponding resistance genes into the environment, with a high potential to return to the human and animal populations by water or irrigated food consumption. Therefore, not only must the presence of AB resistant bacteria be monitored in the different environmental matrices, but targeted treatments should also be developed and implemented at full-scale in the WWTPs, so that the produced wastewater effluents could be safely discharged into the environment and / or reused for other purposes, such as agricultural irrigation. Additionally, and since this study was performed with samples collected from only one WWTP during a narrow period of time, studies with samples collected from multiple WWTPs in different timepoints / seasons will be valuable to strengthen these findings and to answer to the new and exciting scientific question raised by this work: Why is there a significant difference in the transmission of AB resistance genes between the groups of bacteria here distinguished as potentially environmental and potentially pathogenic?

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DATA AVAILABILITY STATEMENT

The whole genome sequencing data files were deposited in GenBank within the BioProject with the SRA accession number PRJNA683808.

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CHAPTER 4

Nanofiltration as an efficient tertiary wastewater treatment: Elimination of total bacteria and antibiotic resistance genes from the discharged effluent of a full-scale wastewater treatment plant

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Micaela Oliveira was involved in all the experimental work presented in this chapter. Inês Leonardo helped in the detection and quantification of the total – live and dead – bacteria by flow cytometry. João Goulão Crespo helped performing the treatment of the discharged effluent samples with the Desal 5 DK nanofiltration membrane. Ana Filipa Silva, Mónica Nunes and Maria Teresa Barreto Crespo supervised all the experimental work performed.

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ABSTRACT

Wastewater reuse for agricultural irrigation still raises important public health issues regarding its safety, due to the increasing presence of emerging contaminants, such as antibiotic resistant bacteria and genes, in the treated effluents. In this paper, the potential for a commercial Desal 5 DK nanofiltration membrane to be used as a tertiary treatment in the wastewater treatment plants for a more effective elimination of these pollutants from the produced effluents was assessed on laboratory scale, using a stainless steel cross-flow cell. The obtained results showed high concentrations of total bacteria and target carbapenem and (fluoro)quinolone resistance genes (bla_{KPC}, bla_{OXA-48}, bla_{NDM}, bla_{IMP}, bla_{VIM}, *qnr*A, *qnr*B and *qnr*S) not only in the discharged, but also in the reused effluent samples, which suggests that their use may not be entirely safe. Nevertheless, the applied nanofiltration treatment achieved removal rates superior to 98 % for the total bacteria and 99.99 % for all the target resistance genes present in both DNA and extracellular DNA fractions, with no significant differences for these microbiological parameters between the nanofiltered and the control tap water samples. Although additional studies are still needed to fully optimize the entire process, the use of nanofiltration membranes seems to be a promising solution to substantially increase the quality of the treated wastewater effluents.

Keywords: Antibiotic resistance; Carbapenem and (fluoro)quinolone resistance; Tertiary wastewater treatments; Nanofiltration; Wastewater reuse

4.1 INTRODUCTION

Water scarcity has been a worldwide problem and a central issue on the international agenda over the last few decades (Mancosu et al., 2015; Mekonnen and Hoekstra, 2016). The agricultural sector alone is responsible for the consumption of about 70 % of the available freshwater on Earth, being this demand expected to continue to grow due to the projected increase of the world population in the coming years (FAO, 2007). This scenario makes wastewater reuse for agricultural irrigation a valuable and sustainable alternative. However, despite this being a practice already implemented in different water-scarce countries around the world (Helmecke et al., 2020), there are still important public health issues regarding its quality and safety, even if it complies with the current legislation on water reuse (Regulation (EU) 2020/741), which fails to account for the presence of contaminants of emerging concern, such as antibiotic (AB) resistant bacteria and genes, in the reclaimed water. In fact, multiple studies already point out for the inefficiency of the conventionally applied wastewater treatments in the removal of AB resistant bacteria and genes from the treated effluents (for discharge and reuse) (Cacace et al., 2019; Lamba and Ahammad, 2017; Mathys et al., 2019; Oliveira et al., 2021, 2020; Pärnänen et al., 2019; Yang et al., 2016). Moreover, their discharge into the environment can degrade the quality of the water bodies, making their subsequent use as potable water sources and for multiple industrial applications difficult (Panagopoulos and Haralambous, 2020a, 2020b). Therefore, the use of an inappropriately treated reclaimed water for agricultural irrigation purposes may result in the contamination of soils, crops and groundwater reservoirs with these micropollutants, posing a direct risk for both the farm workers and crop consumers (Christou et al., 2017; Jaramillo and Restrepo, 2017; Ungureanu et al., 2020).

To improve the quality and safety of the reclaimed water and prevent the harmful effects that may arise from its reuse, several advanced treatment technologies, such as membrane separation processes, ozonation, H_2O_2-derived oxidation, electrochemical

oxidation and sulfate radical-advanced oxidation processes, have been developed and tested for the removal of different emerging contaminants (Garcia-Ivars et al., 2017; González et al., 2015; Sun et al., 2022; Xu et al., 2020). Among them, the use of membrane separation processes, such as ultrafiltration, nanofiltration and reverse osmosis, is currently considered a powerful solution, with nanofiltration being one of the most cost-efficient methods to perform enhanced wastewater treatments, since it represents a good compromise between the required water quality and the energy expenditure to produce it (Garcia-Ivars et al., 2017; González et al., 2015; Mohammad et al., 2015; Xu et al., 2020). Nanofiltration membranes present separation properties between those of ultrafiltration and reverse osmosis membranes, with a pore size in the order of 1 nm, which corresponds to a molecular weight cut-off in the range of 100 to 5,000 Da (Oatley-Radcliffe et al., 2017). Since nanofiltration is expected to be an effective technique for the removal of multiple emerging micropollutants from wastewaters, it can be used to produce high-quality effluents in a more sustainable way than reverse osmosis, due to its higher permeate flux and ability to work at lower pressures, which contributes to a decrease in the energy consumption (Couto et al., 2018; Foureaux et al., 2019; Oatley-Radcliffe et al., 2017). In fact, several recent studies that used nanofiltration membranes as a tertiary wastewater treatment technique show promising results regarding the removal efficiencies of different contaminants of emerging concern, such as pharmaceutically active compounds, endocrine disruptors, personal care products and heavy metals (Cristóvão et al., 2019; Cuhorka et al., 2020; Foureaux et al., 2019; Garcia-Ivars et al., 2017; Xu et al., 2020). However, only a few have already started to address the threat of AB resistance and, in particular, the resistance towards last-line ABs (the last treatment options for patients infected with bacteria resistant to other available ABs), by focusing on the removal efficiencies of both AB resistant bacteria and genes from the treated wastewater effluents (Cristóvão et al., 2021; Slipko et al., 2019). Therefore, the main goals of the present work are as follows: (1) To determine the concentrations of total – live and dead – bacteria and to

assess the occurrence of carbapenem and (fluoro)quinolone resistance genes in the intracellular and extracellular fractions of discharged effluent samples collected from a full-scale wastewater treatment plant (WWTP); (2) To perform an additional nanofiltration treatment step on these discharged effluent samples using a Desal 5 DK nanofiltration membrane and to further address the removal efficiencies of the total – live and dead – bacteria and of the target carbapenem and (fluoro)quinolone resistance genes in the nanofiltered water samples; (3) To compare the concentrations of total – live and dead – bacteria and of the target carbapenem and (fluoro)quinolone resistance genes present in the intracellular and extracellular fractions of the nanofiltered water samples with those found in the reused effluent samples (produced by the WWTP) and in tap water samples (acting as a control of water that was collected and treated in order to assure high enough quality for direct human and animal consumption).

4.2 MATERIALS AND METHODS

4.2.1 WWTP description and sample collection

Samples were collected from a Portuguese full-scale WWTP designed to treat domestic wastewater of approximately 756,000 population equivalents (P.E.) employing the biological aerated filters technology. After the biological treatment step, most of the produced effluent is directly discharged into the Tagus River, with a smaller fraction being filtered through a cartridge filter, disinfected with the addition of sodium hypochlorite and then reused for green park irrigation and street washing purposes. Three biological samples of 10 L each were collected from these two sampling points – discharged and reused effluents – in sterile containers in July of 2020. After collection, all samples were transported to the laboratory under refrigerated conditions and immediately processed upon arrival. The main steps of the wastewater treatment process are shown in Figure S4.1 and the general analytical control parameters of the discharged effluent samples are listed in Table S4.1.

4.2.2 Treatment of the discharged effluent samples with a Desal 5 DK nanofiltration membrane

The nanofiltration experiments performed on the discharged effluent samples were conducted using a Desal 5 DK nanofiltration membrane (GE Water & Process Technologies, Feasterville-Trevose, PA, USA) on a laboratory scale stainless steel cross-flow Sepa CF II Membrane Cell System (GE Water & Process Technologies, Feasterville-Trevose, PA, USA) with an effective membrane area of 54 cm^2. The Desal 5 DK nanofiltration membrane was selected due to its highly hydrophilic character and low molecular weight cut-off, in order to assure high water permeability and the rejection of small analytes and / or biological entities. However, other nanofiltration membranes could have also been tested, namely the NF90 (FilmTec, Edina, MN, USA). A scheme of the cross-flow system used in this study is represented in Figure 4.1, including the Hydra-Cell positive displacement pump, model G-13 (Warner Engineering, INC., Minneapolis, MN, USA), equipped with a variator SEW, used for the circulation of the feed / retentate stream. The installation also comprised pressure transducers installed at the inlet (feed), outlet (retentate) and permeate lines. The permeate flux was determined by measuring the volume of permeate collected in a defined period of time. The temperature was also measured in order to normalize the permeate flux for a reference temperature of 20 °C.

Before use, the nanofiltration membrane was cleaned with distilled water for the removal of any impurities and distilled water was also filtered at 20 bar until a constant flux was achieved, in order to assure an adequate membrane compaction. All experiments were then performed at constant transmembrane pressure (20 bar) conditions and the removal rates of the total – live and dead – bacteria and of the target carbapenem and (fluoro)quinolone resistant genes were calculated using the following equation:

$$Removal\ (\%) = \left(1 - \frac{Cp}{Cf}\right) \times 100,$$

where *Cp* and *Cf* are the concentrations of the total – live and dead – bacteria or of the target carbapenem or (fluoro)quinolone resistance gene in the permeate and feed, respectively. During the time course of filtration, the fouling was negligible and the permeate flux was rather constant, at a rate of 230 L / (m^2.h).

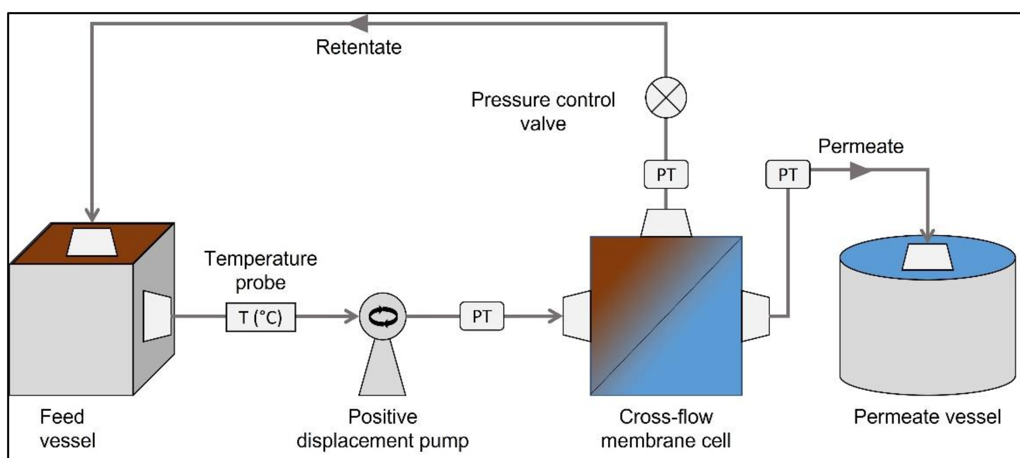

Figure 4.1 | Schematic representation of the cross-flow nanofiltration system used in this study. PT: Pressure transducer.

4.2.3 Detection and quantification of the total – live and dead – bacteria by flow cytometry

The samples obtained from both the discharged and reused effluents were primarily filtered in triplicate through sterile 100 µm pore-size nylon membranes (Merck Millipore, Burlington, NY, USA) to remove larger particles that could interfere with the flow cytometry analysis, whereas the discharged effluent samples submitted to the nanofiltration treatment were directly processed, along with the tap water samples. All samples were stained in triplicate using the LIVE / DEAD™ BacLight™ bacterial viability and counting kit (Invitrogen, Waltham, MA, USA), which contains both the permeant green-fluorescent SYTO™ 9 dye and the impermeant red-fluorescent propidium iodide (PI) dye to distinguish between bacteria with intact and

damaged cell walls, respectively. Briefly, 1 mL of each sample was incubated with 1.5 µL of SYTO™ 9 and 1.5 µL of PI for about 15 min at room temperature, protected from light, and gently mixed before the analysis. From the SYTO™ 9 versus PI plots, which correspond to FL1 (green channel) versus FL3 (red channel), the gates used for the enumeration of live and dead bacteria were defined. Multiple pure cultures of Gram-positive and Gram-negative bacteria were used to confirm and adjust the defined gates for live and dead bacteria, using fresh cultures and bacterial cells treated with isopropanol. All experiments were performed in triplicate on a CyFlow® Space (Sysmex Partec GmbH, Gorlitz, Germany), equipped with a blue laser emitting at 488 nm. Single-color controls were performed for instrument adjustment and the aqueous solution Sheath Fluid (Sysmex Partec GmbH, Gorlitz, Germany), used to assure hydrodynamic focusing, was also analyzed with and without staining to measure the background noise.

4.2.4 Detection and quantification of the target carbapenem and (fluoro)quinolone resistance genes by *TaqMan* multiplex qPCR

First, all samples were filtered in triplicate through 0.22 µm pore-size polyethersulfone filters (Pall Corporation, New York, NY, USA) and the filtration volumes were determined by assuming the clogging of the filters as a measure of, approximately, the same amount of filtered biomass. Therefore, volumes of 50 mL for the discharged effluent samples and 90 mL for the reused effluent samples were filtered. Since there was no clogging of the filters when filtering the discharged effluent samples submitted to the nanofiltration treatment and the tap water samples, the filtered volumes for these two samples were 2,000 mL. After filtration, the filters proceeded for DNA extraction and 15 mL of each filtrate proceeded for precipitation and purification of the eDNA. The DNA of each sample was extracted following the standard protocol from the DNeasy® PowerWater® Kit (Qiagen, Hilden, Germany), whereas the eDNA was precipitated with absolute ethanol and 3 M sodium acetate, as

previously described by (Foote et al., 2012), and purified using the DNeasy® UltraClean® Microbial Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Both DNA and eDNA concentrations and purities were then measured using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). At the end, the detection and quantification of the most clinically relevant and globally distributed carbapenem and (fluoro)quinolone resistance genes was performed using three previously developed *TaqMan* multiplex qPCR assays (Oliveira et al., 2020): (1) *TaqMan* multiplex qPCR 1, designed for the quantification of bla_{KPC} and $bla_{OXA\text{-}48}$-type genes; (2) *TaqMan* multiplex qPCR 2, designed for the quantification of bla_{NDM}, bla_{IMP} and bla_{VIM}-type genes; (3) *TaqMan* multiplex qPCR 3, designed for the quantification of *qnr*A, *qnr*B and *qnr*S-type genes. All experiments were performed in triplicate on a LightCycler 96 Real-Time PCR System (Roche, Basel, Switzerland).

4.2.5 Statistical analysis

The mean values of the concentrations of the total – live and dead – bacteria and of the target carbapenem and (fluoro)quinolone resistance genes in the different samples were compared using multiple one-way analysis of variance tests (ANOVA). For each test, the homogeneity of the variances was previously evaluated with a Levene's test. If the resulting differences were significant, the variances were not considered homogeneous and an ANOVA with the Dunnett T3 post hoc test was performed; If the resulting differences were not significant, the variances were considered homogeneous and an ANOVA with the Tukey post hoc test was performed. These statistical analyses were performed using the SPSS 26 software (IBM, Armonk, NY, USA) and the differences were considered significant at $p < 0.05$.

4.3 RESULTS

4.3.1 Total – live and dead – bacteria present in the different samples

The concentrations of total – live and dead – bacteria present in the different samples are shown in Figure 4.2 and Table S4.2. In the discharged effluent samples, the concentrations of total bacteria were 1.5×10^6 cells / mL – 1.1×10^6 cells / mL of live bacteria and 4.0×10^5 cells / mL of dead bacteria (Figure 4.2; Table S4.2). In the reused effluent samples, the concentrations of total bacteria were 8.0×10^5 cells / mL – 6.1×10^5 cells / mL of live bacteria and 1.9×10^5 cells / mL of dead bacteria, which represents a significant ($p < 0.05$) logarithmic reduction of 0.28 and a removal rate of 47.02 %, regarding the concentrations of total bacteria observed in the discharged effluent samples (Figure 4.2; Tables S4.2 and S4.3). For the discharged effluent samples submitted to the nanofiltration treatment – from now on designated as nanofiltered water samples – the concentrations of total bacteria were 1.9×10^4 cells / mL – 1.3×10^4 cells / mL of live bacteria and 6.2×10^3 cells / mL of dead bacteria, which represents a significant ($p < 0.05$) logarithmic reduction of 1.89 and a removal rate of 98.72 %, regarding the concentrations of total bacteria observed in the discharged effluent samples (Figure 4.2; Tables S4.2 and S4.3). For the tap water samples, the concentrations of total bacteria were 8.8×10^3 cells / mL – 6.9×10^3 cells / mL of live bacteria and 1.8×10^3 cells / mL of dead bacteria, which represents no significant differences ($p > 0.05$), regarding the concentrations of total bacteria observed in the nanofiltered water samples (Figure 4.2; Table S4.2).

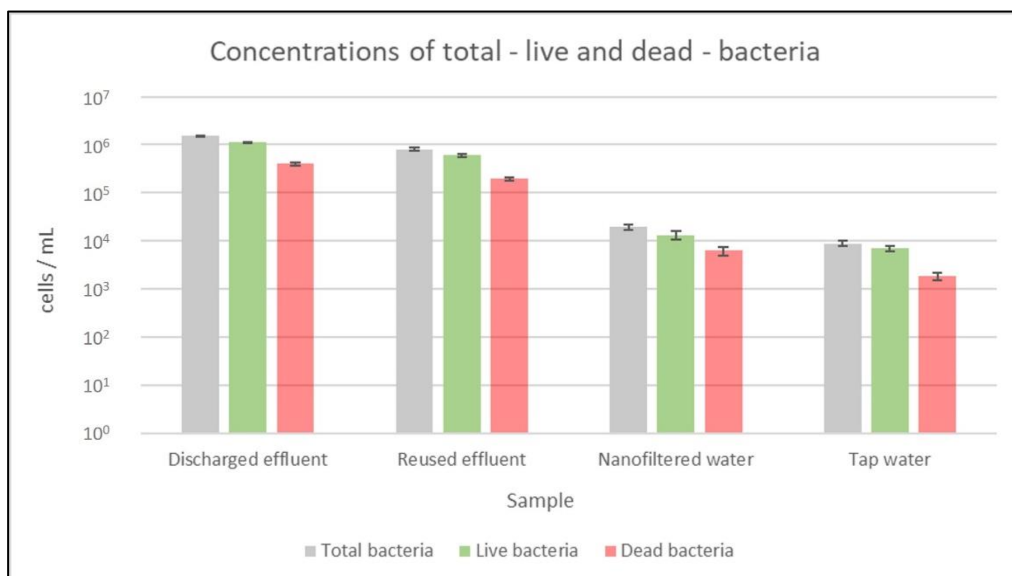

Figure 4.2 | Concentrations of total – live and dead – bacteria in the discharged effluent, reused effluent, nanofiltered water and tap water samples. Values are expressed in cells per milliliter and correspond to the mean ± standard deviation of biological and technical triplicates.

4.3.2 Carbapenem and (fluoro)quinolone resistance genes present in the different samples

The concentrations of the carbapenem and (fluoro)quinolone resistance genes present in the DNA fraction of the different samples are shown in Figure 4.3 and Table S4.4. All target carbapenem and (fluoro)quinolone resistance genes – *bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *qnr*A, *qnr*B and *qnr*S – were detected in the discharged effluent samples (Figure 4.3; Table S4.4). Among them, the most abundant were the *qnr*S and *bla*$_{VIM}$ genes, with concentrations of 5.9 x 10^5 and 1.9 x 10^5 gene copy numbers / mL, respectively (Figure 4.3; Table S4.4). Despite the significant ($p < 0.05$) reduction in their concentrations, five of these genes – *bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{VIM}$, *qnr*B and *qnr*S – were still detected in the reused effluent samples, with concentrations ranging from 3.2 x 10^1 to 1.2 x 10^5 gene copy numbers / mL and removal rates between 42.81 % and 99.77 % regarding their concentrations in the discharged effluent samples (Figure 4.3; Tables

S4.4 and 4.5). On the contrary, none of the target carbapenem and (fluoro)quinolone resistance genes were detected in the nanofiltered water samples, with the removal rates regarding their concentrations in the discharged effluent samples being superior to 99.99 % (Figure 4.3; Tables S4.4 and 4.5). In the control tap water samples, there was also no detection of any of the eight target carbapenem and (fluoro)quinolone resistance genes under study (Figure 4.3; Table S4.4).

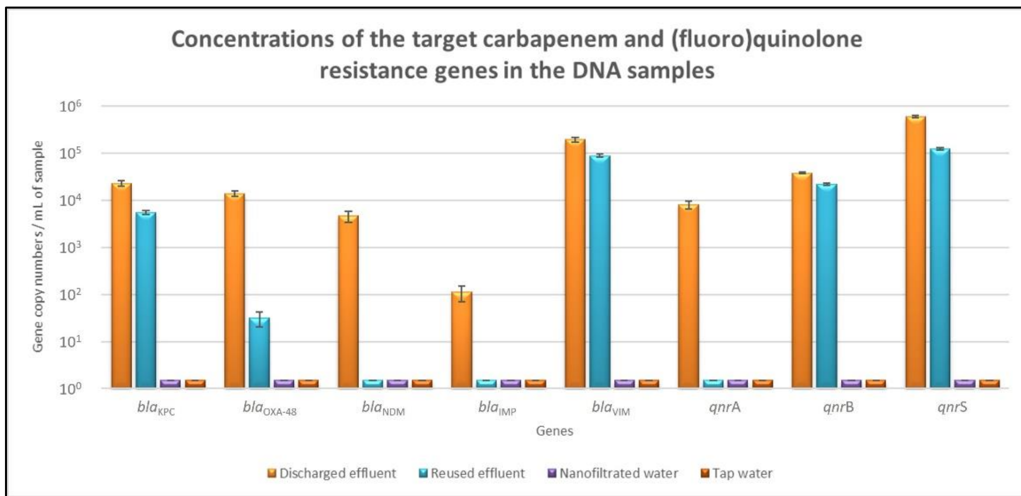

Figure 4.3 | Concentrations of the target carbapenem and (fluoro)quinolone resistance genes in the DNA extracted from the discharged effluent, reused effluent, nanofiltered water and tap water samples. Values are expressed in gene copy numbers per milliliter and correspond to the mean ± standard deviation of biological and technical triplicates.

With regard to the extracellular DNA (eDNA) fraction of the different samples, the concentrations of the carbapenem and (fluoro)quinolone resistance genes are shown in Figure 4.4 and Table S4.6. Two of the target carbapenem and (fluoro)quinolone resistance genes – *bla*$_{\text{VIM}}$ and *qnr*S – were detected in the discharged effluent samples, with concentrations of 1.3×10^3 and 4.3×10^2 gene copy numbers / mL, respectively (Figure 4.4; Table S4.6). Despite the significant ($p < 0.05$) reduction in its concentration, the *qnr*S gene was still detected in the reused effluent samples, with a concentration

of 2.8 x 10^2 gene copy numbers / mL and a removal rate of 34.55 %, regarding its concentration in the discharged effluent samples (Figure 4.4; Tables S4.6 and S4.7). Similar to what was observed in the DNA fraction, none of the target carbapenem and (fluoro)quinolone resistance genes were detected in the eDNA fraction of the nanofiltered water samples, with the removal rates regarding their concentrations in the discharged effluent samples being superior to 99.99 % (Figure 4.4; Tables S4.6 and S4.7). In the control tap water samples, there was also no detection of any of the eight target carbapenem and (fluoro)quinolone resistance genes under study (Figure 4.4; Table S4.6).

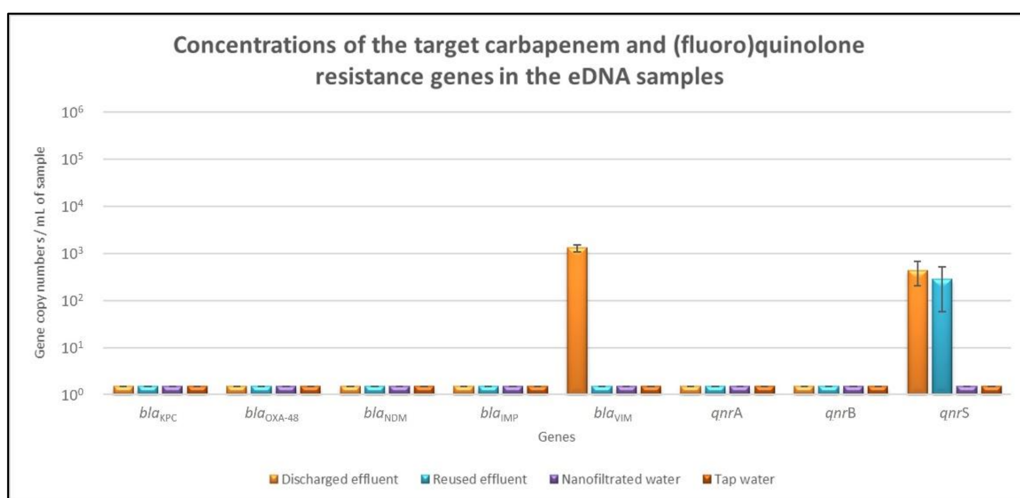

Figure 4.4 | Concentrations of the target carbapenem and (fluoro)quinolone resistance genes in the eDNA precipitated and purified from the discharged effluent, reused effluent, nanofiltered water and tap water samples. Values are expressed in gene copy numbers per milliliter and correspond to the mean ± standard deviation of biological and technical triplicates.

4.4 DISCUSSION

Membrane separation processes are currently considered among the most promising and attractive solutions for the challenge of water quality and wastewater reuse (Shannon et al., 2008). In this study, the effectiveness of the nanofiltration

technique in the removal of total bacteria and AB resistance genes from discharged effluent samples collected in a Portuguese full-scale WWTP was tested in laboratory conditions using a cross-flow system equipped with a commercial Desal 5 DK nanofiltration membrane.

Since the majority of the environmental bacteria still fails to grow on culture media (Alain and Querellou, 2009), a culture-independent flow cytometry viability assay was performed for the quantification of the total bacteria present in the different samples, allowing the quantification not only of the non-cultivable bacteria, but also of the dead bacteria. This is especially relevant since dead bacteria can lyse and release their chromosomal DNA and mobile genetic elements to the environment, which may harbour AB resistant genes that can be later assimilated by other bacteria and bacteriophages via natural transformation. The results show that, despite the significant reduction in the concentrations of total – live and dead – bacteria from the discharged effluent to the reused effluent samples (with a removal rate of 47.02 %), this reduction was even greater from the discharged effluent to the nanofiltered water samples, reaching a removal rate over 98 % and concentrations of total – live and dead – bacteria similar to those observed in the control tap water samples. However, since the Desal 5 DK nanofiltration membranes have a molecular weight cut-off between 150 and 300 Da, it would be expected that all the bacteria present in the discharged effluent samples should be retained by the membrane during the treatment. In fact, the concentrations of total – live and dead – bacteria observed in the nanofiltered water samples can likely be explained by the manipulation of these samples during some of the steps of both their treatment and analysis, where it was not possible to maintain the sterility conditions (for example, after the nanofiltration treatment, when passing through both the permeate collecting tubes and the channels of the flow cytometer). Therefore, as with the tap water samples, which also circulate along the water distribution pipes in non-sterile conditions, it is not possible to obtain a water completely free of bacteria. Furthermore, it is important to mention that the

quantifications of total – live and dead – bacteria observed in the discharged effluent samples should only be considered as an indicative microbiological parameter, since not all bacteria harbour AB resistance genes. Despite this, in 2019, more than half of the *Escherichia coli* isolates and more than a third of the *Klebsiella pneumoniae* isolates reported by European countries to the European Antimicrobial Resistance Surveillance Network (EARS-Net) were resistant to at least one of the AB groups under surveillance and the simultaneous resistance to different AB groups was also frequent (ECDC, 2020). Therefore, once in the WWTPs, these bacteria find the suitable conditions to proliferate and horizontally transfer their AB resistance genes to other bacteria (Guo et al., 2017; Michael et al., 2013; Oliveira et al., 2020; Rizzo et al., 2013), leading to an expected increase in the concentrations of AB resistant bacteria and, consequently, a high percentage of these microorganisms in the discharged effluent samples.

Regarding the presence of the target carbapenem and (fluoro)quinolone resistance genes in the discharged effluent samples, their high concentrations reinforce the increasing resistance that the community has been acquiring to these ABs, which is particularly important and worrying in the case of carbapenems, as they are one of the most important groups of last-line ABs (Oliveira et al., 2020). The results obtained for the DNA fraction (the DNA extracted from the bacterial community cells present in the different samples) show that, despite the significant reduction in the concentrations of the eight target carbapenem and (fluoro)quinolone resistance genes, five of these genes – bla_{KPC}, $bla_{OXA\text{-}48}$, bla_{VIM}, *qnr*B and *qnr*S – were still detected in the reused effluent samples. This emphasizes the inefficiency of the conventional wastewater treatments in the removal of AB resistant bacteria and corresponding resistance genes from the treated effluents, which consequently act as important sources of AB resistance dissemination into the environment and back to the human and animal populations. On the contrary, none of the target carbapenem and (fluoro)quinolone resistance genes were detected in the nanofiltered water samples. Their removal rates were calculated considering the previously determined *TaqMan* multiplex qPCR detection

limit of 10 gene copy numbers per microliter (Oliveira et al., 2020) and were superior to 99.99 % in all cases. This complete removal of the eight target carbapenem and (fluoro)quinolone resistance genes shows that the Desal 5 DK nanofiltration membrane is effective in the elimination of multiple AB resistant bacteria and corresponding resistance genes, highlighting that the bacteria detected in the nanofiltered water samples by the flow cytometry assay were a result of the manipulation of these samples. These results agree with recent studies that evaluated the removal efficiencies of different AB resistance genes from swine wastewaters by nanofiltration (Lan et al., 2019; Liang et al., 2021). As in the present work, the results were promising and verified that, despite the inefficiency of the biological treatments in the removal of these micropollutants, the subsequent nanofiltration treatments led to reductions in the order of 4.98-9.52 logs when compared to raw sewage (Lan et al., 2019), or higher than 99.79 % (Liang et al., 2021). In addition, a pilot scale study on the occurrence of multiple AB resistance genes in a municipal wastewater effluent and their treatment by a nanofiltration unit obtained extremely high rejections of these target contaminants (Cristóvão et al., 2021). It is also interesting to notice that, in both the discharged and reused effluent samples, the concentrations of total bacteria – 1.5×10^6 and 8.0×10^5 cells / mL, respectively – were in the same order of magnitude as the concentrations of some of the target AB resistance genes under study, namely the *qnr*S gene, which presented concentrations of 5.9×10^5 gene copy numbers / mL in the discharged effluent samples and 1.2×10^5 gene copy numbers / mL in the reused effluent samples. At a first glance, these results would suggest that a large percentage of the bacteria present in these samples – about 39 % of the bacteria present in the discharged effluent samples and 15 % of the bacteria present in the reused effluent samples – would harbour at least one of the target carbapenem and (fluoro)quinolone resistance genes. However, it is important to keep in mind that bacteria can harbour not just one, but several plasmids containing resistance determinants. Therefore, multiple copies of the same resistance gene may be located in the same bacteria, which

is in fact commonly observed in bacteria harbouring plasmids containing *qnr* genes (García-Fernández et al., 2009; Jacoby et al., 2014). Thus, if on the one hand it is possible that there were fewer bacteria harbouring the target carbapenem and (fluoro)quinolone resistance genes in our samples than initially thought, on the other hand there may be more multiresistant bacteria, harbouring different plasmids with multiple AB resistance genes. As for the results obtained for the eDNA fraction (the free / extracellular DNA present in the different samples), two of the target carbapenem and (fluoro)quinolone resistance genes – bla_{VIM} and *qnr*S – were detected in the discharged effluent samples and one of them – *qnr*S – was still detected in the reused effluent samples. Similar to what was observed in the DNA fraction, the complete removal of the eight target carbapenem and (fluoro)quinolone resistance genes only occurred during the nanofiltration treatment, since none of them were detected in the nanofiltered water samples. These results are in line with a recent study showing that membranes with a molecular weight cut-off smaller than 5,000 Da can retain more than 99.80 % of the eDNA, both in plasmid and linear forms, with size exclusion as the main retention mechanism (Slipko et al., 2019).

Overall, the results obtained in this study show that the Desal 5 DK nanofiltration membranes have a great potential to be used as a tertiary treatment step in the WWTPs, due to their high removal efficiencies of total – live and dead – bacteria and AB resistance genes. This would allow the production of a reclaimed water with superior quality, which could be used not only more safely in the activities where it is already being used, but also in areas where it is highly needed, such as agricultural irrigation. Nevertheless, additional studies are still required to test the long-term filtration performance of these membranes and to optimize the process under different conditions and contaminant loads. Furthermore, the retentate treatment is also a crucial topic to be addressed in future studies. It might be recycled back to the feed stream of the WWTP without introducing a noticeable charge load on it or, as an alternative, future approaches might consider treating these nanofiltration

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concentrates with advanced oxidation processes, despite the increased costs to the overall treatment that this option will lead to.

4.5 CONCLUSIONS

The practice of wastewater reuse for agricultural irrigation purposes still raises public health issues regarding its quality and safety, due to the inefficiency of the conventional wastewater treatments in the removal of different emerging contaminants from the treated effluents. Among them, the presence of AB resistant bacteria and corresponding resistance genes in the effluents for discharge and reuse stands out as an important threat in most countries, as these streams are direct gateways for their dissemination into the environment and back to the human and animal populations. This study assessed the potential of a commercial nanofiltration membrane to be used as a tertiary treatment in the WWTPs for a more effective elimination of bacteria and AB resistance genes from the produced effluents. Altogether, the obtained results showed an extremely high efficiency in the removal of total bacteria and AB resistance genes from the discharged effluent samples: (1) The concentrations of total bacteria observed in the nanofiltered water samples were significantly lower than those present in the reused effluent samples and similar to the ones detected in the control tap water samples; (2) The concentrations of carbapenem and (fluoro)quinolone resistance genes in the nanofiltered water samples were reduced for values under the detection limit of 10 gene copy numbers per microliter, which is also significantly lower than the concentrations present in the reused water samples and similar to the ones detected in the control tap water samples. Therefore, despite the need for additional studies that test the long-term filtration performance of these membranes, optimize the process under different conditions and contaminant loads and focus on the retentate treatment, Desal 5 DK nanofiltration membranes seem to have great potential to be used as a tertiary treatment step in the WWTPs, allowing the production of a high-quality reclaimed water that can be more safely used

in the activities where it is already being used, but also in other areas, such as agricultural irrigation.

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CHAPTER 5

Discussion and Future work

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5.1 INTRODUCTION

The main objectives of the work presented in this thesis were: (1) To provide a deep insight on the occurrence and persistence of the carbapenem resistome, as well as on the characterization of the corresponding resistant bacteria, from the produced wastewater influents to the reused streams, in two Portuguese full-scale wastewater treatment plants; (2) To address if the application of a final nanofiltration treatment step after the conventional wastewater treatment can be considered an alternative for the production of high-quality effluents for reuse. This study was fundamental not only to demonstrate that the conventionally applied treatments for wastewater reuse are inefficient, being this stream an important and direct gateway for the dissemination of last-line antibiotic resistant bacteria and genes back to the human and animal populations, but also to present an alternative treatment, able to produce effluents free of antibiotic-related pollutants that could be more safely reused in agricultural irrigation. The main achievements are summarized in this section and the proposed future work is also discussed.

5.2 OCCURRENCE AND PERSISTENCE OF THE CARBAPENEM RESISTOME IN TWO PORTUGUESE FULL-SCALE WASTEWATER TREATMENT PLANTS

In this study, the population dynamics of two Portuguese full-scale wastewater treatment plants was characterized along different sampling points, including the reused effluents, in both cellular and extracellular DNA samples. The analysis was performed by high throughput sequencing targeting the 16S rRNA V4 gene region and by three in-house *TaqMan* multiplex qPCR assays that detect and quantify the most clinically relevant and globally distributed carbapenem – bla_{KPC}, $bla_{OXA\text{-}48}$, bla_{NDM}, bla_{IMP}, bla_{VIM} – and (fluoro)quinolone – *qnr*A, *qnr*B, *qnr*S – resistance genes.

The obtained results demonstrated that: (1) The biological treatment is the most important step on shaping the bacterial community composition and on affecting the

concentrations of the target carbapenem and (fluoro)quinolone resistance genes along the wastewater treatment process; (2) Regardless of the different operational designs and treatments applied in the two wastewater treatment plants, carbapenem and (fluoro)quinolone resistance genes persisted at high concentrations in the DNA and extracellular DNA fractions of both discharged and reused effluent samples; (3) The extracellular DNA fraction of the wastewater is an important source of carbapenem and (fluoro)quinolone resistance genes, representing an additional pathway for the dissemination of antibiotic resistance. Altogether, it was not only undoubtedly shown the importance of the wastewater treatment plants and of the environmental sector on the antibiotic resistance spreading cycle, but it was also demonstrated that, for wastewater reuse to become a safe and reliable practice, targeted treatments towards antibiotic resistant bacteria and genes must be developed and implemented in the wastewater treatment plants.

5.3 CHARACTERIZATION OF THE CARBAPENEM RESISTANT BACTERIA PRESENT IN A PORTUGUESE FULL-SCALE WASTEWATER TREATMENT PLANT

In this study, two distinct groups of carbapenem resistant bacteria – potentially environmental and potentially pathogenic – were isolated from wastewater influent and discharged effluent samples collected in a full-scale wastewater treatment plant and then characterized both genotypically, through whole genome sequencing, and phenotypically, by antibiotic susceptibility testing.

The obtained results showed that: (1) Although with a reduction in the observed concentrations between the wastewater influent and the discharged effluent, both potentially environmental and potentially pathogenic carbapenem resistant bacteria were still present at high concentrations in the discharged effluent samples; (2) The potentially environmental carbapenem resistant bacteria presented low detection rates of acquired antibiotic resistance genes, appearing to be resistant to carbapenems and to other antibiotics essentially through intrinsic mechanisms; (3) The potentially

pathogenic carbapenem resistant bacteria presented high detection rates of acquired resistance genes towards carbapenems and other antibiotic classes, and their major antibiotic resistance mechanism appears to be their acquisition and expression; (4) Most of the gene fragments detected by the three in-house *TaqMan* multiplex qPCR assays and detailed in the previous chapter were proven to correspond to complete and functional antibiotic resistance genes that are present in living bacteria, which can horizontally transfer them to other bacteria. Altogether, it was reinforced that the conventionally applied wastewater treatments are clearly inefficient in the elimination of multiple antibiotic resistant bacteria and genes from the treated effluents, which thus act as important vehicles for the dissemination of these micropollutants into the environment, with a high potential to return to the human and animal populations by water or irrigated food consumption. Therefore, not only the occurrence of antibiotic resistant bacteria and genes must be monitored in the different environmental matrices, but targeted treatments should also be developed and implemented in the wastewater treatment plants, so that the produced effluents could be more safely discharged or reused.

5.4 APPLICATION OF A NANOFILTRATION TREATMENT STEP FOR THE PRODUCTION OF HIGH-QUALITY EFFLUENTS FOR REUSE

In this study, the potential for a commercial Desal 5DK nanofiltration membrane to be used as a tertiary treatment step in the wastewater treatment plants for a more effective elimination of the antibiotic resistant bacteria and genes from the produced effluents was access on laboratory scale, using a stainless steel cross-flow cell. The detection and quantification of the total – live and dead – bacteria and of the target carbapenem and (fluoro)quinolone resistance genes were performed before and after the applied treatment (using as control reused wastewater and tap water samples) by flow cytometry and *TaqMan* multiplex qPCR assays, respectively.

The obtained results demonstrated that: (1) The concentrations of total bacteria observed in the nanofiltered water samples were significantly lower than those present in the reused effluent samples and similar to the ones detected in the control tap water samples; (2) The concentrations of both carbapenem and (fluoro)quinolone resistance genes in the nanofiltered water samples were reduced to values bellow the detection limit of one gene copy number per milliliter, which is also significantly lower than the concentrations present in the reused effluent samples and similar to the ones detected in the control tap water samples. Altogether, it was demonstrated the extremely high efficiency of these nanofiltration membranes in the removal of total bacteria and antibiotic resistance genes from discharged effluent samples. Therefore, Desal 5 DK nanofiltration membranes seem to have a great potential to be applied as a tertiary treatment step in the wastewater treatment plants, allowing the production of high-quality effluents that can be more safely used in the activities where they are already being used, but also in areas such as agricultural irrigation.

5.5 FUTURE WORK

5.5.1 Nanofiltration assays at pilot scale

Regarding the nanofiltration treatment of the discharged effluent samples, there is still much work that have to be performed in order to fully optimize the process. For instance, additional studies at pilot scale are required to test the long-term filtration performance of these membranes and to test the operation under different conditions and contaminant loads. Furthermore, the retentate treatment is also a crucial topic to be addressed, since it might be recycled back to the feed stream of the wastewater treatment plant without introducing a noticeable charge load on it or, alternatively, future approaches might consider treating these nanofiltration concentrates applying advanced oxidation processes, despite the increased costs to the overall treatment that this option will lead to.

5.5.2 Microbiological evaluation of crops irrigated with the nanofiltered water

The cultivation of soft fruits and / or vegetables irrigated with the nanofiltered water will allow a microbiological safety evaluation regarding the internalization of the antibiotic resistant bacteria and corresponding resistance genes that could be present in the reclaimed water by the different tissues of these food products. It is expected that the produced reclaimed water does not work as a vehicle of transmission of any of these micropollutants to the cultivated food products and, ultimately, to the crop consumers, proving that, when properly treated, wastewater can be safely reused for agricultural irrigation.

APPENDICES

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SUPPLEMENTARY INFORMATION FROM CHAPTER 2

Table S2.1 | DNA concentrations measured on a Qubit Fluorometer after extraction for use in the high throughput sequencing. The DNA was extracted from the wastewater samples collected at the different sampling points of both WWTPs.

WWTP	Sampling point & Replicate number	Concentration (ng / µL)
WWTP A	Sampling point 1 - Replicate 1	39.0
	Sampling point 1 - Replicate 2	17.7
	Sampling point 1 - Replicate 3	29.4
	Sampling point 2 - Replicate 1	45.6
	Sampling point 2 - Replicate 2	48.0
	Sampling point 2 - Replicate 3	53.2
	Sampling point 3 - Replicate 1	26.2
	Sampling point 3 - Replicate 2	31.8
	Sampling point 3 - Replicate 3	25.8
	Sampling point 4 - Replicate 1	17.2
	Sampling point 4 - Replicate 2	19.5
	Sampling point 4 - Replicate 3	19.3
WWTP B	Sampling point 1 - Replicate 1	39.2
	Sampling point 1 - Replicate 2	47.6
	Sampling point 1 - Replicate 3	40.2
	Sampling point 2 - Replicate 1	13.0
	Sampling point 2 - Replicate 2	10.3
	Sampling point 2 - Replicate 3	18.4
	Sampling point 3 - Replicate 1	13.4
	Sampling point 3 - Replicate 2	11.8
	Sampling point 3 - Replicate 3	13.6
	Sampling point 4 - Replicate 1	6.2
	Sampling point 4 - Replicate 2	6.7
	Sampling point 4 - Replicate 3	9.5

Table S2.2 | DNA concentrations measured on a NanoDrop 1000 Spectrophotometer after extraction for use in the *TaqMan* qPCR assays. The DNA was extracted from the wastewater samples collected at the different sampling points of both WWTPs.

WWTP	Sampling point & Replicate number	Concentration (ng / µL)
WWTP A	Sampling point 1 - Replicate 1	59.14
	Sampling point 1 - Replicate 2	57.21
	Sampling point 1 - Replicate 3	77.28
	Sampling point 2 - Replicate 1	120.65
	Sampling point 2 - Replicate 2	103.98
	Sampling point 2 - Replicate 3	114.49
	Sampling point 3 - Replicate 1	64.57
	Sampling point 3 - Replicate 2	70.92
	Sampling point 3 - Replicate 3	88.73
	Sampling point 4 - Replicate 1	124.24
	Sampling point 4 - Replicate 2	92.62
	Sampling point 4 - Replicate 3	91.32
WWTP B	Sampling point 1 - Replicate 1	50.71
	Sampling point 1 - Replicate 2	42.17
	Sampling point 1 - Replicate 3	54.14
	Sampling point 2 - Replicate 1	41.38
	Sampling point 2 - Replicate 2	48.78
	Sampling point 2 - Replicate 3	75.56
	Sampling point 3 - Replicate 1	65.42
	Sampling point 3 - Replicate 2	50.85
	Sampling point 3 - Replicate 3	64.60
	Sampling point 4 - Replicate 1	36.75
	Sampling point 4 - Replicate 2	30.11
	Sampling point 4 - Replicate 3	40.38

Table S2.3 | eDNA concentrations measured on a NanoDrop 1000 Spectrophotometer after precipitation and purification for use in the *TaqMan* qPCR assays. The eDNA was precipitated and purified from the wastewater samples collected at the different sampling points of both WWTPs.

WWTP	Sampling point & Replicate number	Concentration (ng / µL)
WWTP A	Sampling point 1 - Replicate 1	5.1
	Sampling point 1 - Replicate 2	4.6
	Sampling point 1 - Replicate 3	4.2
	Sampling point 2 - Replicate 1	3.6
	Sampling point 2 - Replicate 2	3.9
	Sampling point 2 - Replicate 3	3.1
	Sampling point 3 - Replicate 1	2.5
	Sampling point 3 - Replicate 2	2.3
	Sampling point 3 - Replicate 3	3.0
	Sampling point 4 - Replicate 1	2.1
	Sampling point 4 - Replicate 2	2.4
	Sampling point 4 - Replicate 3	1.9
WWTP B	Sampling point 1 - Replicate 1	3.9
	Sampling point 1 - Replicate 2	4.2
	Sampling point 1 - Replicate 3	4.9
	Sampling point 2 - Replicate 1	2.6
	Sampling point 2 - Replicate 2	2.7
	Sampling point 2 - Replicate 3	3.2
	Sampling point 3 - Replicate 1	2.4
	Sampling point 3 - Replicate 2	1.9
	Sampling point 3 - Replicate 3	2.2
	Sampling point 4 - Replicate 1	1.6
	Sampling point 4 - Replicate 2	2.1
	Sampling point 4 - Replicate 3	1.8

Table S2.4 | *p* values obtained in the different Levene's Tests performed to test the homogeneity of the variances of the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria along the different sampling points of each WWTP. Differences were considered significant at $p < 0.05$.

Phyla	WWTP A	WWTP B
Actinobacteria	$p = 0.187$	$p = 0.215$
Bacteroidetes	$p = 0.409$	$p = 0.462$
Firmicutes	$p = 0.180$	$p = 0.136$
Proteobacteria	$p = 0.464$	$p = 0.271$

Table S2.5 | *p* values obtained in the different Levene's Tests performed to test the homogeneity of the variances of the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria between the corresponding sampling points of both WWTPs. Differences were considered significant at $p < 0.05$.

Phyla	Sampling point 1	Sampling point 2	Sampling point 3	Sampling point 4
Actinobacteria	$p = 0.330$	$p = 0.981$	$p = 0.173$	$p = 0.227$
Bacteroidetes	$p = 0.062$	$p = 0.607$	$p = 0.463$	$p = 0.131$
Firmicutes	$p = 0.623$	$p = 0.162$	$p = 0.756$	$p = 0.323$
Proteobacteria	$p = 0.812$	$p = 0.196$	$p = 0.455$	$p = 0.204$

Table S2.6 | *p* values obtained in the different Levene's Tests performed to test the homogeneity of the variances of the 16S rRNA, bla_{KPC}, bla_{VIM}, *qnr*B and *qnr*S genes along the different sampling points of each WWTP. Differences were considered significant at $p < 0.05$.

Gene	WWTP A	WWTP B
16S rRNA	$p = 0.002$	$p = 0.006$
bla_{KPC}	$p = 0.002$	$p = 0.030$
bla_{VIM}	$p = 0.022$	$p = 0.048$
*qnr*B	$p = 0.002$	$p = 0.001$
*qnr*S	$p = 0.001$	$p = 0.003$

Table S2.7 | Relative abundances of the eight most represented phyla present in the different sampling points of both WWTPs. Values are expressed in percentages and correspond to the mean of biological triplicates.

	WWTP A				WWTP B			
Phyla	**Sampling point 1**	**Sampling point 2**	**Sampling point 3**	**Sampling point 4**	**Sampling point 1**	**Sampling point 2**	**Sampling point 3**	**Sampling point 4**
Actinobacteria	10.16 %	1.68 %	12.80 %	11.16 %	5.19 %	19.75 %	19.85 %	22.50 %
Bacteroidetes	2.42 %	30.51 %	34.99 %	32.25 %	7.52 %	8.69 %	10.03 %	8.45 %
Candidatus Saccharibacteria	-	0.22 %	0.20 %	-	-	3.90 %	4.46 %	5.26 %
Chloroflexi	-	0.40 %	0.14 %	0.51 %	-	4.05 %	2.88 %	4.18 %
Euryarchaeota	1.01 %	-	0.25 %	0.60 %	0.39 %	-	-	-
Firmicutes	45.26 %	9.73 %	3.70 %	5.12 %	33.76 %	12.33 %	10.71 %	13.56 %
Fusobacteria	1.18 %	-	-	-	0.97 %	-	-	-
Planctomycetes	-	-	-	0.32 %	-	-	-	0.83 %
Proteobacteria	36.62 %	55.07 %	45.58 %	46.14 %	51.37 %	39.16 %	39.97 %	35.14 %
Synergistetes	0.44 %	0.58 %	-	-	0.13 %	-	-	-
Tenericutes	-	-	-	-	-	1.28 %	1.19 %	-
Verrucomicrobia	0.85 %	0.37 %	0.67 %	1.05 %	0.46 %	1.86 %	1.75 %	0.94 %
Others	2.05 %	1.45 %	1.68 %	2.85 %	0.22 %	8.97 %	9.16 %	9.14 %

Table S2.8 | *p* values obtained in the different one-way analysis of variance tests (ANOVA) performed to compare the mean values of the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria between the corresponding sampling points of both WWTPs. Differences were considered significant at $p < 0.05$.

Phyla	Sampling point 1	Sampling point 2	Sampling point 3	Sampling point 4
Actinobacteria	$p = 0.005$	$p = 0.000$	$p = 0.001$	$p = 0.000$
Bacteroidetes	$p = 0.001$	$p = 0.000$	$p = 0.000$	$p = 0.000$
Firmicutes	$p = 0.000$	$p = 0.006$	$p = 0.000$	$p = 0.000$
Proteobacteria	$p = 0.001$	$p = 0.000$	$p = 0.005$	$p = 0.000$

Table S2.9 | *p* values obtained in the different one-way analysis of variance tests (ANOVA) performed to compare the mean values of the phyla Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria before and after the biological treatment step of each WWTP. Differences were considered significant at $p < 0.05$.

Phyla	WWTP A	WWTP B
Actinobacteria	$p = 0.000$	$p = 0.000$
Bacteroidetes	$p = 0.000$	$p = 0.023$
Firmicutes	$p = 0.000$	$p = 0.000$
Proteobacteria	$p = 0.000$	$p = 0.000$

Table S2.10 | Relative abundances of the eight most represented genera present in the different sampling points of both WWTPs. Values are expressed in percentages and correspond to the mean of biological triplicates.

	WWTP A				WWTP B			
Genera	**Sampling point 1**	**Sampling point 2**	**Sampling point 3**	**Sampling point 4**	**Sampling point 1**	**Sampling point 2**	**Sampling point 3**	**Sampling point 4**
Acidovorax	-	3.67 %	4.66 %	4.82 %	-	-	-	-
Acinetobacter	13.42 %	5.52 %	-	-	26.75 %	3.23 %	2.85 %	2.54 %
Arcobacter	-	3.14 %	-	-	4.25 %	4.57 %	5.98 %	5.47 %
Bacteroides	-	-	-	-	-	-	1.58 %	2.97 %
Bifidobacterium	3.94 %	-	-	-	-	-	-	-
Blautia	3.64 %	-	-	-	3.28 %	-	-	-
Chloroflexus	-	-	-	-	-	1.48 %	-	1.75 %
Chryseobacterium	-	4.52 %	-	-	-	-	-	-
Cloacibacterium	-	5.41 %	-	-	-	-	-	-
Clostridium	-	-	-	-	-	3.56 %	2.49 %	3.78 %
Comamonas	3.69 %	2.73 %	-	-	3.81 %	-	-	-
Corynebacterium	-	-	-	-	-	1.39 %	1.03 %	1.67 %
Faecalibacterium	5.12 %	-	-	-	3.90 %	-	-	-
Flavobacterium	-	7.55 %	21.50 %	20.22 %	-	-	1.23 %	-
Glaciecola	-	-	4.91 %	1.78 %	-	-	-	-

Haliscomenobacter	-	-	-	-	-	1.48 %	-	-
Jeotgalibaca	6.18 %	-	-	-	4.73 %	-	-	-
Leucobacter	-	-	-	2.27 %	-	-	-	-
Limnohabitans	-	-	4.55 %	3.35 %	-	-	-	-
Moraxella	6.14 %	-	-	-	3.32 %	-	-	-
Mycobacterium	-	-	-	-	-	1.62 %	1.61 %	1.78 %
Novosphingobium	-	-	4.41 %	-	-	-	-	-
Pseudarcicella	-	-	2.32 %	-	-	-	-	-
Pseudomonas	-	-	-	3.40 %	-	-	-	-
Rhodoferax	-	-	-	-	-	2.69 %	2.20 %	1.88 %
Serpentinomonas	-	16.25 %	2.03 %	2.19 %	-	-	-	-
Streptococcus	7.57 %	-	-	-	3.36 %	-	-	-
Thiocystis	-	-	2.54 %	1.93 %	-	-	-	-
Others	50.30 %	51.21 %	53.08 %	60.04 %	46.61 %	79.97 %	81.02 %	78.16 %

Table S2.11 | Specificity of each *TaqMan* multiplex qPCR reaction. Results correspond to the mean of technical triplicates.

	***TaqMan* Multiplex qPCR 1**	***TaqMan* Multiplex qPCR 2**	***TaqMan* Multiplex qPCR 3**
bla_{KPC}	-	No detection	No detection
bla_{OXA-48}	-	No detection	No detection
bla_{NDM}	No detection	-	No detection
bla_{IMP}	No detection	-	No detection
bla_{VIM}	No detection	-	No detection
*qnr*A	No detection	No detection	-
*qnr*B	No detection	No detection	-
*qnr*S	No detection	No detection	-

Table S2.12 | Average abundances of the 16S rRNA, *bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *qnr*A, *qnr*B and *qnr*S genes in the DNA extracted from the bacterial community cells of the different sampling points of both WWTPs. Values are expressed in gene copy numbers per milliliter and correspond to the mean ± standard deviation of biological and technical triplicates.

	WWTP A				**WWTP B**			
Gene	**Sampling point 1**	**Sampling point 2**	**Sampling point 3**	**Sampling point 4**	**Sampling point 1**	**Sampling point 2**	**Sampling point 3**	**Sampling point 4**
16S rRNA	9.3E+09 (±1.3E+09)	1.1E+09 (±8.7E+07)	1.8E+08 (±4.2E+07)	2.3E+08 (±2.8E+07)	6.6E+09 (±9.4E+08)	7.5E+07 (±2.3E+07)	4.3E+07 (±1.1E+07)	2.9E+07 (±5.6E+06)
bla$_{KPC}$	1.7E+06 (±3.9E+05)	1.8E+05 (±1.9E+04)	2.2E+04 (±9.1E+03)	2.4E+04 (±6.1E+03)	1.5E+05 (±3.1E+04)	1.4E+03 (±6.2E+02)	3.9E+02 (±1.1E+02)	7.2E+02 (±2.0E+02)
bla$_{OXA-48}$	1.7E+05 (±2.9E+04)	b.d.l.	b.d.l.	9.2E+03 (±2.8E+03)	4.0E+05 (±4.6E+04)	b.d.l.	b.d.l.	b.d.l.
bla$_{NDM}$	1.1E+05 (±2.4E+04)	b.d.l.	b.d.l.	b.d.l.	7.6E+04 (±2.4E+04)	b.d.l.	b.d.l.	b.d.l.
bla$_{IMP}$	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.
bla$_{VIM}$	7.2E+06 (±1.5E+06)	4.1E+05 (±7.7E+04)	3.8E+04 (±8.7E+03)	6.9E+04 (±1.3E+04)	9.4E+05 (±3.8E+05)	1.6E+04 (±3.0E+03)	7.7E+03 (±7.1E+02)	3.2E+03 (±5.8E+02)
*qnr*A	4.8E+03 (±1.0E+03)	b.d.l.	b.d.l.	b.d.l.	8.8E+02 (±2.0E+03)	b.d.l.	b.d.l.	b.d.l.
*qnr*B	1.6E+06 (±3.1E+05)	4.6E+04 (±5.6E+03)	8.0E+03 (±1.4E+03)	6.9E+03 (±1.2E+03)	2.3E+04 (±3.6E+03)	1.5E+03 (±3.3E+02)	1.6E+03 (±1.9E+02)	2.2E+03 (±3.3E+02)
*qnr*S	2.7E+07 (±7.3E+06)	5.2E+05 (±9.1E+04)	8.4E+04 (±1.9E+04)	6.4E+04 (±1.6E+04)	3.7E+05 (±1.1E+05)	2.4E+04 (±7.8E+03)	2.4E+04 (±3.4E+03)	2.8E+04 (±4.2E+03)

b.d.l. below detection limit

Table S2.13 | p values obtained in the different one-way analysis of variance tests (ANOVA) performed to compare the mean values of the 16S rRNA, bla_{KPC}, bla_{VIM}, *qnr*B and *qnr*S genes before and after the biological treatment step of each WWTP. Differences were considered significant at $p < 0.05$.

Gene	WWTP A	WWTP B
16S rRNA	$p = 0.022$	$p = 0.026$
bla_{KPC}	$p = 0.078$	$p = 0.058$
bla_{VIM}	$p = 0.060$	$p = 0.179$
*qnr*B	$p = 0.046$	$p = 0.022$
*qnr*S	$p = 0.087$	$p = 0.064$

Table S2.14 | Average abundances of the 16S rRNA, *bla*$_{KPC}$, *bla*$_{OXA-48}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{VIM}$, *qnr*A, *qnr*B and *qnr*S genes in the eDNA of the different sampling points of both WWTPs. Values are expressed in gene copy numbers per milliliter and correspond to the mean ± standard deviation of biological and technical triplicates.

	WWTP A				**WWTP B**			
Gene	**Sampling point 1**	**Sampling point 2**	**Sampling point 3**	**Sampling point 4**	**Sampling point 1**	**Sampling point 2**	**Sampling point 3**	**Sampling point 4**
16S rRNA	1.6E+05 (±2.4E+04)	2.6E+06 (±1.2E+06)	1.1E+05 (±1.9E+04)	2.9E+06 (±2.7E+05)	1.7E+06 (±6.0E+05)	2.3E+05 (±5.4E+05)	2.7E+04 (±1.3E+04)	9.1E+04 (±5.1E+04)
bla$_{KPC}$	b.d.l.	b.d.l.	b.d.l.	b.d.l.	5.7E+02 (±2.8E+02)	1.8E+02 (±8.4E+01)	b.d.l.	b.d.l.
bla$_{OXA-48}$	5.6E+02 (±2.5E+02)	b.d.l.	b.d.l.	b.d.l.	9.0E+02 (±2.7E+02)	b.d.l.	b.d.l.	b.d.l.
bla$_{NDM}$	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.
bla$_{IMP}$	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.
bla$_{VIM}$	1.2E+05 (±1.1E+05)	3.8E+03 (±1.2E+03)	3.5E+03 (±1.6E+03)	2.1E+04 (±5.6E+03)	5.2E+03 (±2.5E+03)	1.9E+03 (±8.4E+02)	5.3E+03 (±3.2E+03)	3.2E+03 (±2.1E+03)
*qnr*A	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.	b.d.l.
*qnr*B	2.6E+02 (±2.2E+02)	3.9E+02 (±1.9E+02)	1.6E+02 (±4.1E+01)	2.5E+02 (±6.3E+01)	5.7E+02 (±3.1E+02)	b.d.l.	b.d.l.	b.d.l.
*qnr*S	9.3E+02 (±2.0E+02)	3.9E+03 (±1.4E+03)	6.7E+02 (±2.5E+02)	1.2E+03 (±5.1E+02)	1.0E+04 (±2.4E+03)	4.6E+02 (±1.5E+02)	b.d.l.	b.d.l.

b.d.l. below detection limit

SUPPLEMENTARY INFORMATION FROM CHAPTER 3

Table S3.1 | Information about the sequence and amplicon size of the primers and probes used in each *TaqMan* qPCR reaction.

Gene	Primer / Probe	Sequence (5' – 3')	Amplicon (bp)
	KPC Fw	GACGGAAAGCTTACAAAAACTGACA	
bla_{KPC}	KPC Rv	CTTGTCATCCTTGTTAGGCG	259
	KPC Probe	FAM-ACTGGGCAGTCGGAGACAAAACCGGA-BHQ1	
	OXA-48 Fw	TTCGAATTTCGGCCACGG	
bla_{OXA-48}	OXA-48 Rv	CATCAAGTTCAACCCAACCG	204
	OXA-48 Probe	HEX-CCATGCTGACCGAAGCCAATGGTG-BHQ1	
	NDM Fw	GGTTTGGCGATCTGGTTTTC	
bla_{NDM}	NDM Rv	ATCCAGTTGAGGATCTGGGC	181
	NDM Probe	FAM-CGGGGCAGTCGCTTCCAACGGTT-BHQ1	
	IMP Fw	GGAATAGAGTGGCTTAAYTCTC	
bla_{IMP}	IMP Rv	CAAGCTTCTATATTTGCGTCACC	275
	IMP Probe	HEX-TTATCCAGGCCCGGGACACAC-BHQ1	
	VIM Fw	GATGAGTTGCTTTTGATTGATACAGC	
bla_{VIM}	VIM Rv	CGCCCGAAGGACATCAA	153
	VIM Probe	ROX-ACGCACTTTCATGACGACCGCGTC-BHQ2	

Table S3.2 | Information about the concentrations and volumes of mastermix, primers, probes, DNA templates and nuclease free water used in each *TaqMan* qPCR reaction.

***TaqMan* qPCR**	**Mix reaction**
TaqMan multiplex qPCR 1: *bla*$_{KPC}$ and *bla*$_{OXA-48}$ genes	10 µL SensiFAST Probe No-ROX Kit (Bioline, London, UK) 400 nM *bla*$_{KPC}$ forward and reverse primers; 200 nM *bla*$_{OXA-48}$ forward and reverse primers 100 nM *bla*$_{KPC}$ and *bla*$_{OXA-48}$ *TaqMan* probes 50 ng *bla*$_{KPC}$ and *bla*$_{OXA-48}$ DNA templates Nuclease free water (to make 20 µL)
TaqMan multiplex qPCR 2: *bla*$_{NDM}$, *bla*$_{IMP}$ and *bla*$_{VIM}$ genes	10 µL SensiFAST Probe No-ROX Kit (Bioline, London, UK) 200 nM *bla*$_{NDM}$, *bla*$_{IMP}$ and *bla*$_{VIM}$ forward and reverse primers 100 nM *bla*NDM and *bla*IMP *TaqMan* probes; 10 nM *bla*$_{VIM}$ *TaqMan* probe 50 ng *bla*$_{NDM}$, *bla*$_{IMP}$ and *bla*$_{VIM}$ DNA templates Nuclease free water (to make 20 µL)

Table S3.3 | Concentrations of carbapenem resistant bacteria in the wastewater influent and discharged effluent after 24 h incubation at 30 °C or 42 °C. Values are expressed in colony forming units per milliliter.

		Incubation at 30 °C		**Incubation at 42 °C**	
		CFU / mL influent	**CFU / mL effluent**	**CFU / mL influent**	**CFU / mL effluent**
Biological sample 1	Plate 1	8.10E+04	5.60E+03	1.70E+03	1.14E+02
	Plate 2	9.00E+04	4.70E+03	2.20E+03	1.02E+02
	Plate 3	9.50E+04	4.90E+03	2.10E+03	1.25E+02
Biological sample 2	Plate 4	1.02E+05	3.50E+03	1.80E+03	1.41E+02
	Plate 5	1.05E+05	3.60E+03	1.40E+03	1.38E+02
	Plate 6	1.13E+05	4.30E+03	1.70E+03	1.38E+02
Biological sample 3	Plate 7	9.10E+04	7.10E+03	8.00E+02	1.04E+02
	Plate 8	9.50E+04	6.60E+03	3.00E+02	1.14E+02
	Plate 9	9.00E+04	8.00E+03	4.00E+02	1.08E+02
Mean ± standard deviation		9.58E+04 ± 9.58E+03	5.37E+03 ± 1.74E+03	1.38E+03 ± 7.82E+02	1.20E+02 ± 1.63E+01

Table S3.4 | Species identification by 16S rRNA gene sequencing and screening of carbapenem resistance genes in the carbapenem resistant bacteria isolated from the wastewater influent and discharged effluent samples at 30 °C.

Sampling point	Species identification	Total isolated	Carbapenem resistance genes				
			bla $_{KPC}$	***bla*** $_{OXA-48}$	***bla*** $_{NDM}$	***bla*** $_{IMP}$	***bla*** $_{VIM}$
Wastewater influent	*Aeromonas caviae*	7	3	-	-	-	-
	Aeromonas salmonicida	1	-	-	-	-	-
	Aeromonas veronii	7	3	-	-	-	-
	Pseudomonas entomophila	1	-	-	-	-	-
	Pseudomonas fluorescens	3	-	-	-	-	-
	Pseudomonas fragi	1	-	-	-	-	-
	Pseudomonas lundensis	1	-	-	-	-	-
	Pseudomonas migulae	1	-	-	-	-	-
	Pseudomonas psychrophila	1	-	-	-	-	-
	Pseudomonas putida	7	2	-	-	-	-
	Pseudomonas syringae	1	-	-	-	-	-
	Raoultella ornithinolytica	1	-	-	-	-	-
	Total	32	8	0	0	0	0
Discharged effluent	*Acinetobacter pittii*	1	-	-	-	-	-
	Aeromonas caviae	1	-	-	-	-	-
	Aeromonas veronii	8	-	-	-	-	-
	Chromobacterium rhizoryzae	3	-	-	-	-	-
	Pseudomonas entomophila	1	-	-	-	-	-
	Pseudomonas fluorescens	2	-	-	-	-	-
	Pseudomonas fragi	2	-	-	-	-	-
	Pseudomonas monteilii	1	-	-	-	-	-
	Pseudomonas psychrophila	1	-	-	-	-	-
	Pseudomonas putida	6	-	-	-	-	-
	Total	26	0	0	0	0	0

Table S3.5 | Species identification by 16S rRNA gene sequencing and screening of carbapenem resistance genes in the carbapenem resistant bacteria isolated from the wastewater influent and discharged effluent samples at 42 °C.

Sampling point	Species identification	Total isolated	Carbapenem resistance genes				
			***bla*KPC**	***bla*OXA-48**	***bla*NDM**	***bla*IMP**	***bla*VIM**
Wastewater influent	*Acinetobacter baumannii*	6	4	-	-	-	2
	Acinetobacter pittii	5	3	1	-	-	1
	Citrobacter amalonaticus	1	1	-	-	-	-
	Citrobacter freundii	2	2	-	-	-	-
	Enterobacter asburiae	2	1	-	-	-	1
	Escherichia coli	6	4	-	-	-	2
	Klebsiella pasteurii	1	1	-	-	-	-
	Klebsiella pneumoniae	2	1	1	-	-	1
	Raoultella ornithinolytica	2	2	-	-	-	-
	Total	27	19	2	0	0	7
Discharged effluent	*Acinetobacter baumannii*	5	-	-	-	-	-
	Acinetobacter pittii	1	-	-	-	-	-
	Aeromonas veronii	2	-	-	-	-	-
	Citrobacter amalonaticus	1	1	-	-	-	-
	Citrobacter freundii	1	1	-	-	-	1
	Escherichia coli	4	4	-	-	-	-
	Klebsiella pneumoniae	8	8	-	-	-	-
	Total	22	14	0	0	0	1

Table S3.6 | Taxonomic identification of the carbapenem resistant bacteria isolated from the discharged effluent samples at 30 °C using BLASTn, SpeciesFinder 2.0 and KmerFinder 3.2.

BLASTn	SpeciesFinder 2.0	KmerFinder 3.2
Pseudomonas fluorescens	*Pseudomonas fluorescens* *	*Pseudomonas fluorescens*
Pseudomonas fragi	*Pseudomonas fragi*	*Pseudomonas fragi*
Aeromonas caviae	*Aeromonas enteropelogenes*	*Aeromonas caviae*
Aeromonas veronii	*Aeromonas salmonicida*	*Aeromonas veronii*
Chromobacterium rhizoryzae	*Chromobacterium haemolyticum*	*Chromobacterium rhizoryzae*
Pseudomonas entomophila	*Pseudomonas putida* *	*Pseudomonas entomophila*
Pseudomonas monteilii	*Pseudomonas putida* *	*Pseudomonas monteilii*
Pseudomonas psychrophila	*Pseudomonas fragi*	*Pseudomonas fragi*
Pseudomonas putida	*Pseudomonas putida* *	*Pseudomonas* sp. URMO17WK12:I11
Acinetobacter pittii	*Acinetobacter* sp. 151 *	*Acinetobacter oleivorans*

* - Fail in the confidence of the result

Table S3.7 | Taxonomic identification of the carbapenem resistant bacteria isolated from the discharged effluent samples at 42 °C using BLASTn, SpeciesFinder 2.0 and KmerFinder 3.2.

BLASTn	SpeciesFinder 2.0	KmerFinder 3.2
Aeromonas veronii	*Aeromonas veronii*	*Aeromonas veronii*
Klebsiella pneumoniae	*Klebsiella pneumoniae* *	*Klebsiella pneumoniae*
Acinetobacter baumannii	Acinetobacter sp. 148	*Acinetobacter baumannii*
Acinetobacter pittii	*Acinetobacter* sp. 159	*Acinetobacter pittii*
Escherichia coli	*Shigella sonnei*	*Escherichia coli*
Citrobacter amalonaticus	*Citrobacter amalonaticus*	*Citrobacter* sp. Y3
Citrobacter freundii	*Citrobacter freundii*	*Citrobacter portucalensis*

* - Fail in the confidence of the result

Table S3.8 | Acquired AB resistance genes present in the whole genomes of the carbapenem resistant bacteria isolated from the discharged effluent samples at 30 °C.

Bacteria	β-lactams	Aminoglycosides	(Fluoro)quinolones	Macrolides	Phenicol	Rifampicin	Sulphonamides	Trimethoprim
Acinetobacter oleivorans	bla_{ADC-25}, $bla_{OXA-304}$	-	-	-	-	-	-	-
Aeromonas caviae	bla_{MOX-4}, bla_{MOX-6}	-	-	-	-	-	*sul*1	*dfr*A15
Aeromonas veronii	*amp*S, bla_{FOX-2}, bla_{MOX-3}, bla_{OXA-1}, bla_{OXA-10}, *cph*A1, *cph*A4, *cph*A7	*aac(3)-IId*, *aac(6')-Ib*, aac(6')-*Ib3*, *aac(6')-Ib-cr*, *aad*A1, *aph(3')-Ia*, *aph(3'')-Ib*, *aph(6)-Id*	*aac(6')-Ib-cr*	*mph*A	*cat*B3	ARR-3	*sul*1, *sul*2	-
Chromobacterium rhizoryzae	-	-	-	-	-	-	-	-
Pseudomonas entomophila	-	-	-	-	-	-	-	-
Pseudomonas fluorescens	-	-	-	-	-	-	-	-
Pseudomonas fragi	-	-	-	-	-	-	-	-
Pseudomonas fragi	-	-	-	-	-	-	-	-
Pseudomonas monteilii	-	-	-	-	-	-	-	-
Pseudomonas sp. URMO17WK12:I11	-	-	-	-	-	-	-	-

Table S3.9 | Acquired AB resistance genes present in the whole genomes of the carbapenem resistant bacteria isolated from the discharged effluent samples at 42 °C.

Bacteria	β-lactams	Aminoglycosides	Colistin	(Fluoro)quinolones	Fosfomycin
Acinetobacter baumannii	bla_{ADC25}, bla_{OXA-10}, bla_{OXA-24}, bla_{OXA-98}	*aad*A1, *aac(6')-Ib-cr*, *aac(6')-Ib3*	-	*aac(6')-Ib-cr*	-
Acinetobacter pittii	bla_{ADC-25}, $bla_{OXA-255}$, $bla_{OXA-506}$, $bla_{OXA-564}$	-	-	-	-
Aeromonas veronii	*amp*S, *cph*A4, *cph*A7	-	-	-	-
Citrobacter portucalensis	$bla_{CMY-129}$, bla_{KPC-2}, bla_{SHV-12}, bla_{TEM-1A}, bla_{TEM-1C}, bla_{TEM-40}, $bla_{TEM-150}$, $bla_{TEM-171}$, bla_{VIM-1}	*aad*A1, *aac(6')-Ib-cr*, *aac(6')-Ib3*, *aph(3')-XV*	-	*aac(6')-Ib*-cr, *qnr*B6	*fos*A7
Citrobacter sp. Y3	bla_{KPC-2}, bla_{OXA-1}, bla_{OXA-2}, bla_{TEM-1A}, bla_{TEM-1C}, bla_{TEM-40}, $bla_{TEM-150}$, $bla_{TEM-171}$	*aac(6')-Ib-cr*, *aac(6')-Ib3*	-	*aac(6')-Ib-cr*, *oqx*A, *oqx*B	-
Escherichia coli	bla_{KPC-3}, bla_{OXA-9}, bla_{TEM-1A}	*aac(6')-Ib*, *aac(6')-Ib-cr*, *aad*A1, *aad*A2b, *ant(2'')-Ia*, *aph(3'')-Ib*, *aph(6)-Id*	*mcr-9*	*aac(6')-Ib-cr*, *qnr*A1	-
Klebsiella pneumoniae	bla_{KPC-3}, bla_{OXA-9}, $bla_{SHV-101}$, bla_{TEM-1A}	*aad*A1, *aac(6')-Ib*, *aac(6')-Ib-cr*, *aph(3'')-Ib*, *aph(6)-Id*	-	*aac(6')-Ib*-cr, *oqx*A, *oqx*B	*fos*A

Table S3.9 (cont.) | Acquired AB resistance genes present in the whole genomes of the carbapenem resistant bacteria isolated from the discharged effluent samples at 42 °C.

Bacteria	Macrolides	Phenicol	Sulphonamides	Tetracyclines	Trimethoprim
Acinetobacter baumannii	*mph*E, *msr*E	-	-	-	-
Acinetobacter pittii	-	-	-	-	-
Aeromonas veronii	-	-	-	*tet*E	-
Citrobacter portucalensis	-	*cat*B2	*sul*1	*tet*A	*dfr*A1
Citrobacter sp. Y3	-	*cat*B3	*sul*1	-	-
Escherichia coli	-	-	*sul*1, *sul*2	-	*dfr*A14
Klebsiella pneumoniae	-	-	*sul*2	-	*dfr*A14

SUPPLEMENTARY INFORMATION FROM CHAPTER 4

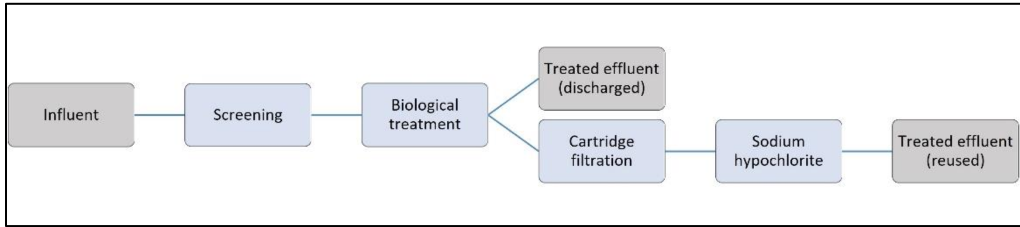


Figure S4.1 | Main steps of the wastewater treatment applied in the full-scale WWTP selected for this study.

Table S4.1 | General analytical control parameters of the discharged effluent samples collected for this study.

pH	TSS[1]	COD[2]	BOD_5[3]	Nitrogen	Nitrates	Phosphorus	Chlorides	Coliforms
7.6	29 mg / L	110 mg / L O_2	25 mg / O_2	32 mg / L N	4.1 mg / L N	1.972 mg / L P	180 mg / L Cl	1.30E+06 NMP / 100 mL

[1] Total suspended solids

[2] Chemical oxygen demand

[3] Biological oxygen demand (5 days)

Table S4.2 | Concentrations of total – live and dead – bacteria present in the discharged effluent, reused effluent, nanofiltered water and tap water samples. Values are expressed in cells per milliliter and correspond to the mean ± standard deviation of biological and technical triplicates.

	Discharged effluent	Reused effluent	Nanofiltered water	Tap water
Total bacteria	1.5E+06 (±5.5E+04)	8.0E+05 (±6.2E+04)	1.9E+04 (±2.7E+03)	8.8E+03 (±1.1E+03)
Live bacteria	1.1E+06 (±2.8E+04)	6.1E+05 (±4.4E+04)	1.3E+04 (±2.7E+03)	6.9E+03 (±8.2E+02)
Dead bacteria	4.0E+05 (±2.8E+04)	1.9E+05 (±1.8E+04)	6.2 E+03 (±1.2E+03)	1.8E+03 (±3.2E+02)

Table S4.3 | Logarithmic reductions and removal rates of the total – live and dead – bacteria from the discharged effluent samples to the reused effluent and nanofiltered water samples.

	Discharged effluent to reused effluent		Discharged effluent to nanofiltered water	
	Logarithmic reduction	**Removal rate**	**Logarithmic reduction**	**Removal rate**
Total bacteria	0.28	47.02 %	1.89	98.72 %
Live bacteria	0.26	45.41 %	1.93	98.82 %
Dead bacteria	0.31	51.52 %	1.81	98.44 %

Table S4.4 | Concentrations of the target carbapenem and (fluoro)quinolone resistance genes present in the DNA fraction of the discharged effluent, reused effluent, nanofiltered water and tap water samples. Values are expressed in gene copy numbers per milliliter and correspond to the mean ± standard deviation of biological and technical triplicates.

Gene	Discharged effluent	Reused effluent	Nanofiltered water	Tap water
bla_{KPC}	2.3E+04 (±2.9E+03)	5.5E+03 (±5.9E+02)	b.d.l.	b.d.l.
bla_{OXA-48}	1.4E+04 (±1.7E+03)	3.2E+01 (±1.1E+01)	b.d.l.	b.d.l.
bla_{NDM}	4.6E+03 (±1.2E+03)	b.d.l.	b.d.l.	b.d.l.
bla_{IMP}	1.1E+02 (±4.1E+01)	b.d.l.	b.d.l.	b.d.l.
bla_{VIM}	1.9E+05 (±2.4E+04)	8.8E+04 (±7.6E+03)	b.d.l.	b.d.l.
*qnr*A	8.1E+03 (±1.6E+03)	b.d.l.	b.d.l.	b.d.l.
*qnr*B	3.8E+04 (±1.3E+03)	2.2E+04 (±1.2E+03)	b.d.l.	b.d.l.
*qnr*S	5.9E+05 (±3.3E+04)	1.2E+05 (±6.7E+03)	b.d.l.	b.d.l.

b.d.l. below detection limit

Table S4.5 | Removal rates of the target carbapenem and (fluoro)quinolone resistance genes from the discharged effluent samples to the reused effluent and nanofiltered water samples in the DNA fraction.

Genes	Discharged effluent to reused effluent	Discharged effluent to nanofiltered water
bla_{KPC}	75,83 %	> 99.99 %
bla_{OXA-48}	> 99.99 %	> 99.99 %
bla_{NDM}	> 99.99 %	> 99.99 %
bla_{IMP}	98,66 %	> 99.99 %
bla_{VIM}	53,97 %	> 99.99 %
*qnr*A	> 99.99 %	> 99.99 %
*qnr*B	42,81 %	> 99.99 %
*qnr*S	79,01 %	> 99.99 %

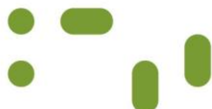
Table S4.6 | Concentrations of the target carbapenem and (fluoro)quinolone resistance genes present in the eDNA fraction of the discharged effluent, reused effluent, nanofiltered water and tap water samples. Values are expressed in gene copy numbers per milliliter and correspond to the mean ± standard deviation of biological and technical triplicates.

Gene	Discharged effluent	Reused effluent	Nanofiltered water	Tap water
bla_{KPC}	b.d.l.	b.d.l.	b.d.l.	b.d.l.
bla_{OXA-48}	b.d.l.	b.d.l.	b.d.l.	b.d.l.
bla_{NDM}	b.d.l.	b.d.l.	b.d.l.	b.d.l.
bla_{IMP}	b.d.l.	b.d.l.	b.d.l.	b.d.l.
bla_{VIM}	1.3E+03 (±2.4E+02)	b.d.l.	b.d.l.	b.d.l.
*qnr*A	b.d.l.	b.d.l.	b.d.l.	b.d.l.
*qnr*B	b.d.l.	b.d.l.	b.d.l.	b.d.l.
*qnr*S	4.3E+02 (±2.3E+02)	2.8E+02 (±2.3E+02)	b.d.l.	b.d.l.

b.d.l. below detection limit

Table S4.7 | Removal rates of the target carbapenem and (fluoro)quinolone resistance genes from the discharged effluent samples to the reused effluent and nanofiltered water samples in the eDNA fraction.

Genes	Discharged effluent to reused effluent	Discharged effluent to nanofiltered water
bla$_{KPC}$	-	-
bla$_{OXA-48}$	-	-
bla$_{NDM}$	-	-
bla$_{IMP}$	-	-
bla$_{VIM}$	> 99.99 %	> 99.99 %
*qnr*A	-	-
*qnr*B	-	-
*qnr*S	34.55 %	> 99.99 %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