Universidade do Minho

Escola de Engenharia

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Characterization of microbial populations in a wastewater treatment plant focusing on *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Dissertação de Mestrado
Mestrado em Bioengenharia

Trabalho realizado sob a orientação da

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Janeiro de 2015

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Título da Dissertação: Characterization of microbial populations in a wastewater treatment plant focusing on *Staphylococcus aureus* and *Pseudomonas aeruginosa*

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Ano de Conclusão: 2015

Designação do Mestrado: Mestrado em Bioengenharia

Universidade do Minho, ___ / ___ / _____

Assinatura: _____

ACKNOWLEDGEMENTS

By accomplishing successfully this work, I would like to acknowledge everyone that was involved in its development and execution, either directly or indirectly.

To Dr. Ana Nicolau and Dr. Maria Olívia Pereira, I would like to express my gratitude, not only for the way I was guided throughout these months, but also for all scientific knowledge instructed.

To Ana Margarida, Diana Alves, and Susana Lopes, I specially thank, for the pleasant way they supported me, for all the patience, and for the time they made available to teach me tirelessly and thoroughly; assisting me in every doubt or concern that arose during the thesis.

To Ana Silva, I am thankful for her companionship, over those laboratory working hours, and endless support, as well as Maria João, Tânia Rodrigues and Rita Seixo.

I also would like to thank to all my Biofilm laboratory coworkers, for the availability and appreciation that they manifested since the beginning, especially to MOP Group, and NC Group.

To all my friends, but in particularly to Guilhermina Carriche, Nuno Machado, Ana Sofia Pereira, Cristina Domingues, Rui Pereira I thank for all the talks, suggestions, patience, and great affection.

Finally, I am grateful to my parents, and my sister, for always being there for me, helping me throughout this journey, cheering me up and supporting me incessantly.

ABSTRACT

Wastewater treatment plants (WWTP) have been regarded as favorable sites for the development and dissemination of antibiotic resistant bacteria (ARB). These bacteria are able to contact with human beings, either directly or indirectly, possibly leading to infections with dire consequences.

The main goal of this study was the evaluation of the efficacy of a WWTP disinfection system (through ultraviolet radiation) at reducing the bacterial load present at the final effluent. Two common pathogenic bacteria - *Staphylococcus aureus* and *Pseudomonas aeruginosa* – were used as indicators of the removal ability of the treatment system. Different colonies of both bacteria were subsequently isolated in order to inspect their antibiotic susceptibility and capacity of biofilm formation.

Four samples were collected during March and June from three different WWTP sites (raw effluent and effluents before and after disinfection). The isolation of *P. aeruginosa* and *S. aureus* was accomplished through the use selective growth media and biochemical confirmation tests. The susceptibility of the selected bacteria to antibiotics was determined through the modified Kirby-Bauer disk diffusion method. Lastly, the ability of the isolated bacteria to form biofilm was investigated using the crystal violet assay, and the number of viable and cultivable biofilm cells through colony counting.

The efficacy of the disinfection treatment in the reduction of bacterial load in the final effluent was effective for *S. aureus*, but not for the removal of *P. aeruginosa.* Twenty-four *P. aeruginosa* and thirty-six *S. aureus* isolate were obtained. The majority of the colonies of both species were susceptible to the tested antibiotics, with the exception of two: ciprofloxacine, with eight *P. aeruginosa* resistant bacteria, and rifampicine, with thirteen isolates of *S. aureus* showing resistance. For both bacteria, the resistant isolates were found mainly in the samples collected before and after the disinfection (BD and AD). The ability of the isolated bacteria to form biofilm was variable, but generally, *P. aeruginosa* isolates achieved a greater biofilm mass.

In conclusion, the efficacy of the WWTP, and namely of the disinfection step, was clearly low if one takes as indicator of its performance the removal ability of *P. aeruginosa*. Besides that, the presence of resistant isolates in BD and AD samples shows that the disinfection is not effective in elimination of ARB, allowing their survival and release to the environment. Due to the great variation of formed biofilm, in both bacteria, it was not possible to establish a relationship between the amount of biofilm formation and the sites of WWTP where the isolates bacteria were collected. No relation was found between a level of resistance to antibiotics and a high capacity of biofilm formation.

RESUMO

As Estações de Tratamento de Águas Residuais (ETAR) têm sido consideradas como locais favoráveis para o desenvolvimento e disseminação de bactérias resistentes aos antibióticos (ARB) para o ambiente.Estas bactérias são capazes de entrar em contacto com o ser humano, directa ou indirectamente, podendo levar a uma infeção com consequências graves.

Este estudo teve como principal objectivo a avaliação da eficiência de uma ETAR com um sistema de desinfecção (através da radiação ultravioleta) na redução da carga bateriana presente no efluente final. Duas bactérias patogénicas comuns - *Staphylococcus aureus* e *Pseudomonas aeruginosa* - foram usadas como indicadores da capacidade de remoção do sistema de tratamento. Posteriormente, diferentes colónias de ambas as bactérias foram isoladas de forma a inspecionar as suas capacidades de formação de biofilme e suceptibilidade aos antibióticos.

Quatro amostras foram recolhidas durante Março e Junho de três localizações diferentes da ETAR (efluente bruto e os efluentes antes e depois da desinfecção). O isolamento *P. aeruginosa* e *S. aureus* foram realizadas através do uso de meios selectivos de crescimento e de testes bioquímicos para confirmação. A susceptibilidade da bactéria aos antibióticos foi determinada através do método de difusão em disco Kirby-Bauer modificado. Por fim, a capacidade de formação de biofilme das bactérias isoladas foi analisada usando o método cristal violeta, e o número de células de biofilmes viáveis e cultiváveis através da contagem de colónias.

A eficácia do tratamento de desinfecção na redução da carga bacteriana do efluente final foi eficaz na remoção *S. aureus* mas não na remoção *P. aeruginosa*. Foram isolados com sucesso vinte e quatro *P. aeruginosa* e trinta e seis *S. aureus*. A maioria das colónias de ambas as espécies foram susceptíveis aos antibióticos testados, à exceção de dois antibióticos: ciprofloxacina, com oito isolados *P. aeruginosa* resistentes, e rifampicina, com treze isolados de *S. aureus* resistentes. Em ambas as bactérias, a localização dos isolados resistentes, mostraram uma presença significativa nas amostras antes e depois da desinfecticção (AD e BD). A capacidade de formação de biofilme dos isolados de ambas as bactérias variou mas, na gobalidade, os isolados *P. aeruginosa* obtiveram uma maior formação de biofilme.

Em conclusão, a eficácia da ETAR, e nomeadamente a etapa da desinfecção foi claramente reduzida se se tiver em conta, como indicador do seu desempenho, a capacidade de remoção *P. aeruginosa*. Além disso, a presença de isolados resistentes nas amostras BD e AD mostrou que a desinfecção não é eficaz na eliminação de ARB, permitindo a sua sobrevivência e libertação para o meio ambiente. Devido à grande variação de biofilme formado, em ambas as bactérias, não foi possível estabelecer uma relação entre quantidade de biofilme formado e os locais da ETAR em que as bactérias isoladas foram recolhidas. Nenhuma relação foi observada entre o nível de resistência aos antibióticos e a elevada capacidade de formação de biofilme.

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ABREVIATIONS

AD	After Disinfection
AMK	Amikacin
ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistant Genes
ATS	Antimicrobial Susceptibility Testing
BD	Before Disinfection
CA	Cetrimide agar
CHF	Chloramphenicol
CIP	Ciprofloxacin
CFU	Colony Forming Units
CV	Crystal Violet
EUCAST	European Committee on Antimicrobial Susceptibility Testing
EPS	Extracellular Polymeric Substances
GENT	Gentamicin
I	Intermediate
MSA	Mannitol Salt Agar
MHA	Mueller Hinton Agar
PIP	Piperacillin
RS	Raw Sewage
R	Resistant
RIF	Rifampicin
S	Susceptible

TETRA	Tetracycline
TOB	Tobymicin
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UV	Ultraviolet
WWTP	Wastewater Treatment Plant

CHAPTER I| INTRODUCTION

1.1. Background

Wastewater treatment plants (WWTP) have a crucial role in protecting public health and in the preservation of water resources. However, WWTP are a main source of release of antibiotics into the environment, being considered as one of the "hotspots" for potential evolution and spread of antibiotic resistance into the environment. The increase in bacterial resistance often drives to an increase in the duration of the treatment and expensive costs.

Unfortunately, the current WWTP are not prepared for the effective elimination of antibiotics and other chemicals as well as pathogenic microorganisms, being therefore important to focus on optimizing the WWTP and on developing new treatments for the removal of these organisms and products. Certain bacteria species known as "ESKAPE" pathogens present in hospital and care unit environments are being a source of concern in WWTP. These bacteria, major causes of nosocomial infections and known to have a high abundance of multi-resistances, are common in the sewage due to the entrance of hospital sewages in the urban WWTP. If the WWTP is not able to eliminate these microorganisms, they will be released into the environment, probably with new resistances, putting at risk the health publishes. For that, it is pivotal to inspect the ability of WWTP to remove pathogenic and antibiotics resistant bacteria in order to obtain insights about the performance of these treatment systems in this particular goal.

1.2. Main objectives

This work aimed to analyze the efficiency of a WWTP with a tertiary treatment disinfection system in reducing the bacterial load, and particularly antibiotic resistant bacteria. *Staphylococcus aureus* and *Pseudomonas aeruginosa,* two common pathogenic bacteria, were selected as indicators of the efficiency of removal through treatment with ultraviolet radiation. Moreover, it was aimed to isolate different strains of both bacteria in order to evaluate two virulence through the analysis of their susceptibility towards different antibiotics and their ability to form biofilms.

1.3. Thesis Organization

The present dissertation is organized in five chapters:

In the first chapter, it is presented and contextualized the general work subject. Likewise, the main goals of the project are defined, as well as it organization throughout the dissertation.

The second chapter presents an introduction about the impact of antibiotics resistant bacteria on human health and environment. The concept of antibiotics resistant bacteria and the definition of biofilm formation are also presented. A description of the relationship between antibiotics resistance and history of the antibiotics is given, and an approach of the way in which both bacteria and antibiotics resistant genes may disseminate to the environment. The concept of WWTP is introduced as well as the main steps associated with the treatment of residual water. The role of wastewater treatment plants in the development, dissemination and fight against bacteria and antibiotics resistant genes are also referred. Finally, the pathogenic microorganisms that were the target of this study are introduced.

The third chapter presents the methods and materials used throughout the execution of the experimental work. The period of sampling and the collected sites of samples in WWTP are described in this section, as well as used methods to enumeration of cultivable bacteria. All isolation of target bacteria steps are presents, since the filtration of samples to the selective media and biochemistry tests. In this part, all the isolation and confirmation work carried out, particularly in the case of *P. aeruginosa*, is described and justified. Lastly, the procedure for biofilm formation and antimicrobial susceptibility testing of all isolates are described.

Chapter four both presents and discusses the obtained results. During this phase, the efficiency of the WWTP was evaluated, namely on what concerns the variations of the total bacterial load, the densities of *P. aeruginosa* and *S. aureus*. At this point, the influence of temperature to distinguish *P. aeruginosa* from other Pseudomonas species is also discussed. The morphological characteristics of the selected isolates were described. Lastly, the antibiotic resistance profile and capacity of biofilm formation of the isolates were evaluated and discussed.

Finally, the fifth chapter presents the principal conclusions of this thesis and suggestions about future work.

CHAPTER II| BIBLIOGRAPHIC REVIEW

2.1. Impact of antibiotic-resistant bacteria and antibiotics in human health and in environment.

Large amounts of antibiotics for human use are released in municipal sewage (Bouki et al., 2013; Sim et al., 2010). Many antibiotics are not completely metabolized and therefore enter in the sewer through the feces (Hirsch et al., 1999). The intentional disposal of unused medicine (Kümmerer, 2003) and veterinary use also contribute to the quantities of antibiotics found in sewage, as well as the runoff from agricultural applications (Díaz-Cruz et al., 2003; Le-Minh et al., 2010).

The biggest concern with the release of antibiotics into the environment is related to the development of antibiotic-resistant genes (ARG) and antibiotic-resistant bacteria (ARB). The increase in ARB in aquatic environments is increasing leading to the creation of selective pressures on natural bacteria species (Alpay-Karaoglu et al., 2007; Kümmerer, 2004).

The transfer of the ARB to humans may occur through several ways: water, food, plants and through manure when it is used as fertilizer (Dolliver and Gupta, 2008; Salyers, 2002). Various resistances to antibiotics are commonly found in all pathogenic and normal flora bacteria which are present in the human intestinal tract (Baquero et al., 2008; Vignesh et al., 2012).

At the environmental level, antibiotics can affect the evolution of the structure of a community, which consequently influences the ecological function of the ecosystem of the water (Aminov and Mackie, 2007; Thiele-Bruhn and Beck, 2005). Ciprofloxacin is an example of a harmful antibiotic to the environment (Kim and Aga, 2007). Its mixture with two more pharmacological products, triclosan (antisetic) and tergite NP10 (surfactant), in the aquatic environment leads to a significant impact on the rate of production of biomass and in the community structure of algae (Wilson et al., 2003).

2.2. Antibiotic resistant bacteria

As it is generally known, all living organisms struggle to adapt to the environment in order to be fit to survive (Alanis, 2005). So, it should not be a surprise for the human being the fact that bacteria have demonstrated a capacity to support and still adapt to the environment, leading to the development of different mechanisms of resistance to antibiotics (Alanis, 2005). Because of this, many strains of bacteria have become resistant and in some cases multi-resistant to antibiotics leading to their ineffectiveness in many cases (Alanis, 2005). A species is denominated antibiotic-

resistant if it is able to function, survive or persist in the presence of higher concentrations of an antimicrobial agent than others species (Smith et al., 1994).

According to Džidić et al. (2008), the increase of antibiotic resistance was due to a combination of selective pressures that enhanced the transmission of resistant organisms. Of all these factors, the most important and worrying is the continued selective pressure exerted by the routinely used antibiotics, which leads to elimination of strains sensitive and consequently a significant increase in resistant strains (Berger-Bächi, 2002; Kolář et al., 2001).

The biology of antibiotic resistance can be divided into two sections: genetic aspects and biochemical aspects (Alanis, 2005; Džidić et al., 2008). The derived-genetic resistance can be intrinsic or acquired. Intrinsic or natural resistance stems from an inherent factor structural or functional associated to an entire bacterial species (Ammor et al., 2008) where they are resistant without any additional genetic alteration (Normark and Normark, 2002). Acquired resistance to antibiotics can occur by mutations or by horizontal gene transfer (Ammor et al., 2008; Normark and Normark, 2002).

Bacteria can become resistant to antibiotics due to five biochemical mechanisms of resistance: enzymatic modification, target modification, active efflux pumps, reduced antimicrobial permeability and bypass of synthetic pathways (Bonomo and Gill, 2005; McKeegan et al., 2002; Normark and Normark, 2002; Stewart and William Costerton, 2001a). The production of biofilm through the phenotypic adaptation is another form of intrinsic resistance (Ascenzi, 2005; Kumar and Pandit, 2012).

2.2.1. Biofilm formation

More than 60 years after the first report on biofilms, they are still a concern in food, environmental and biomedical fields, among others (Flint et al., 1997; Maukonen et al., 2003; Sihorkar and Vyas, 2001; Zobell, 1943). A definition of biofilm has evolved over the last 25 years and in current days, according to Donlan and Costerton (2002), biofilm is defined as "A microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription".

Biofilms can be composed by a population that developed from a unique species or a community derived from mixed microbial species (Davey and O'Toole, 2000; Mah and O'Toole,

2001). Biofilms are highly hydrated (98% of the total biofilm mass) and are composed by microorganisms (prokaryotic and eukaryotic unicellular organisms), multivalent cations, inorganic particles and extracellular polymeric substances (EPS) (Donlan and Costerton, 2002; Flemming, 1991; Wingender et al., 1999). In the majority of biofilms, the percentage of microorganisms is less than 10% of the dry mass, while the matrix can account for over 90% (Flemming and Wingender, 2010). EPS consist not only of polysaccharides, but also proteins, nucleic acids and lipids. The composition of EPS varies between species and growth conditions, and chemical communication, like quorum sensing (QS), between cells in the community stimulates its formation and secretion. QS is important in biofilm formation process and a mechanism used by cells to query extracellular environment (Renner and Weibel, 2011). EPS are responsible for the morphology and internal structure of biofilms, including surface pores and channels (Flemming and Wingender, 2010). Moreover, they can provide a matrix which allows to maintain cells position for a longer period of time in comparison to the planktonic mode (Lewandowski and Evans, 2000). The organisms associated to biofilms grow more slowly than planktonic organisms, probably because the cells are more limited by nutrients, and also because of oxygen depletion (Donlan, 2000).

Bacteria can exist in one of two types of population: planktonic or in biofilm. However in natural aquatic ecosystems more than 99.9% of the bacteria grow in biofilms on wide variety of surfaces (Donlan and Costerton, 2002). Adaptation of biofilm structure for survival in varying environments, comes from an ancient and integral component of the prokaryotic life cycle (Hall-Stoodley et al., 2004).

Bacteria that are embedded in biofilms enjoy several advantages when compared to the planktonic cells. One of the advantages is the ability of the matrix to capture and concentrate minerals and nutrients from the environment, as already mentioned (Beveridge et al., 1997; Flemming, 1991) . Multispecies biofilms can form stable microconsortia, develop physiochemical gradients and intense cell–cell communication (Flemming and Wingender, 2010). Biofilms are also an ideal place for horizontal gene transfer, since cells are maintained in close proximity to each other, and can therefore exchange genetic information (Flemming et al., 2007). Another advantage of biofilms is protection from a wide array of environmental challenges, such as UV exposure, metal toxicity, acid exposure, dehydration, salinity, predation by protozoa, bacterivorous microorganisms and bacteriophages, phagocytosis and, the most worrying for human, several antibiotics and antimicrobial agents (Espeland and Wetzel, 2001; Flemming, 1991; Huq et al.,

2008; Mah and O'Toole, 2001; McNeill and Hamilton, 2003; Shirtliff et al., 2002; Stewart and William Costerton, 2001b; Teitzel and Parsek, 2003).

Since bacteria living in biofilms have a greater resistance to antibiotics when compared to planktonic cells, and pathogenic bacteria are able to produce biofilms, this phenomena is considered a serious concern for the medical community. (Mah and O'Toole, 2001). Biofilms can form on medical devices, such as catheters or implants, and they can also cause infections on human surfaces such as teeth, skin, and the urinary tract (Hall-Stoodley et al., 2004; Hatt and Rather, 2008; Mah and O'Toole, 2001). In addition to medical equipment, various studies reveal the presence of biofilms in the food industry and in water distribution systems. These biofilms can be responsible for infections to humans, especially those belonging to risk groups (Chmielewski and Frank, 2003; Huq et al., 2008; Lee Wong, 1998; Piriou et al., 1997; Szewzyk et al., 2000; WHO, 2006).

The architecture of biofilms reveals that biofilm formation is a complex developmental process that involves several stages, each with unique characteristics (Davies, 2003; McCarty et al., 2014). The process of biofilm formation, considered as an endless cycle, is characterized by five stages (Figure 1): (i) reversible attachment of cells to surfaces; (ii) irreversible attachment; (iii) maturation I; (iv) maturation II; and (v) dispersion (Davies, 2003; Sauer et al., 2002).

Bacteria initially move to a living or non-living surface and attach to it. This corresponds to the first and critical step of biofilm formation (Sauer et al., 2002). Bacteria use a variety of extracellular organelles and proteins for sensing and attaching to the surfaces, such as flagella, pili and outer membrane proteins (Bullitt and Makowski, 1995; Thomas et al., 2004). The second stage comprises the irreversible attachment when bacteria secretes EPS that facilitate adhesion between cells and surfaces (Flemming and Wingender, 2010). The third stage corresponds to the first maturation of biofilms. Cells adsorbed on surfaces replicate and produce EPS, leading to formation

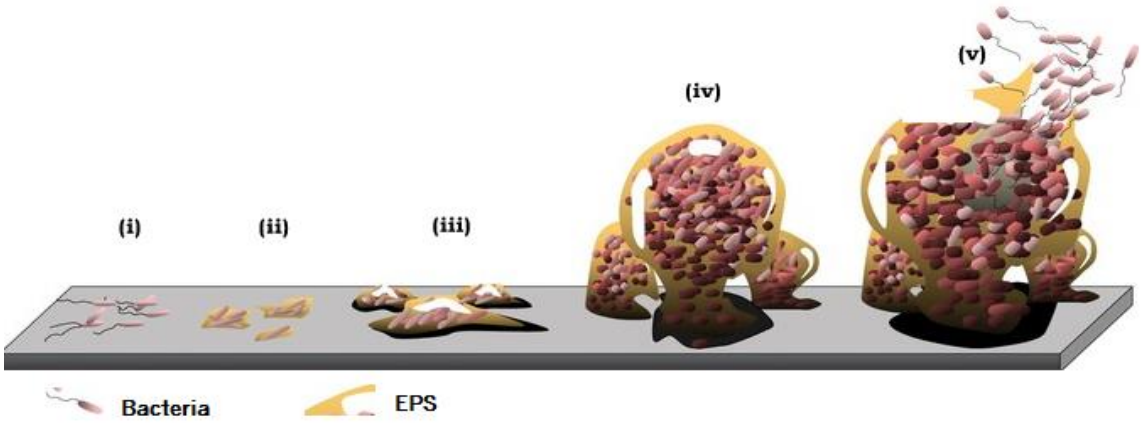

Figure 1| General overview of bacterial biofilm development. (i) reversible attachment of cells to surfaces; (ii) irreversible attachment; (iii) maturation I; (iv) maturation II; and (v) dispersion (adapted from Monroe (2007).

of microcolonies (Karatan and Watnick, 2009; Renner and Weibel, 2011). In the second maturation of biofilm, the fourth stage, the cells continue dividing and the EPS accumulates. In this stage the biofilm is capable of extending from the surface, building a three-dimensional mushroom-shaped structure (Hall-Stoodley et al., 2004). These structures are interspersed with fluid-filled channels which act as a circulatory system, allowing for the exchange of nutrients and waste products with the surrounding environment. The colonies in this mature biofilm are complex and highly differentiated. Numerous microenvironments exist within the biofilm where pH, oxygen concentration, nutrient availability and cell density differ (Kaplan, 2010). After the maturation of the biofilm, some cells detach from the biofilm and disperse (stage v) into the environment with the aim of colonizing other regions (Renner and Weibel, 2011; Stoodley et al., 2002). The dispersal process can be complex, involving numerous environmental signals, signal transduction pathways and effectors. This is important since it contributes to biological dispersion, bacterial survival, and disease transmission (Kaplan, 2010).

The transition from planktonic growth to biofilm involves multiple regulatory networks, which result in gene expression changes causing a temporal reorganization of cellular physiology (Monds and O'Toole, 2009; O'Toole et al., 2000; Prigent-Combaret et al., 2001). Thus, bacteria growing in a biofilm have phenotypical, biochemical, and morphological differences from their planktonic form (Prigent-Combaret et al., 2001).

2.3. Relationship between antimicrobial resistance and the history of antibiotics

Throughout history, there has been an ongoing battle between the human being and the microorganisms that cause infections and diseases. From the Black Death, tuberculosis, malaria, among others, these microorganisms have affected the human population, causing morbidity and mortality.

The use of substances with antimicrobial activity for the treatment of wounds and some diseases dates back thousands of years before the "Age of Antibiotics", used by the ancient Chinese civilization, as well as by the Egyptian and Roman (Moellering, 1995). However, most of these substances were too toxic for applications in other locations. Over time, microbiology skills and techniques were developed which led to the discovery of the first natural antibiotic, in 1928, by Alexander Fleming – the penicillin.

This episode marked the beginning of the "Age of Antibiotics", but only years later, penicillin was introduced for therapeutic purposes (Moellering, 1995). During the "Age of Antibiotics", from 1940 to 1960, there was a considerable number of new antimicrobial agents used in clinical practice and it was believed that infectious diseases could be definitely controlled and prevented (Bockstael and Aerschot, 2009). However, this euphoria was of short duration, since the bacteria able to evolve, expressing various forms of resistance (Livermore, 2003; Tenover, 2006), becoming clear that the victory against bacterial pathologies was an illusion (Kim, 2013).

Throughout years, despite the efforts to control or even eliminate the bacteria, they have always managed to get around the situation in order to endure and prosper. Bacteria are survivors who inhabit the earth for more than four billion years, even in some environments where human life would be impossible.

According to Harbottle et al. (2006), resistance genes and the mechanisms of resistance existed long before the discovery and use of antibiotics. An example was the discovery in glacial samples obtained from the Canadian Arctic archipelago of bacteria resistant to antibiotics over 2000 years old (Dancer et al., 1997).

2.4. Spread of antibiotic resistant bacteria and genes in the environment

The recycling of municipal water for industrial, agricultural and urban non-potable use is an increasingly important component of the management practices of water resources in many parts of the world (Exall, 2004; Wintgens et al., 2005). However, the reuse of treated effluent increases the range of scenarios of human and environmental exposure to ARB (Le-Minh et al., 2010).

Water is not just a means of dissemination of ARB between animal and human populations, but also a route in which ARG are introduced in natural ecosystems. In these systems, non-pathogenic bacteria can serve as a reservoir of resistant genes (Baquero et al., 2008).

According to Baquero et al. (2008), there are four main genetic reactors, places where genetic evolution occurs, in which resistance to antibiotics may be developed:

- First reactor: made by human and animal microbiota, with hundreds of bacterial species involved and where antibiotics exert their action.
- Second reactor: involves hospitals and care centers, farms or anywhere where there are susceptible individuals exposed to bacterial exchange.

- Third reactor: corresponds to sewage or other biological waste arising in the second reactor. This reactor includes lagoons and treatment plants, ideal places for bacteria to have the opportunity to mix and react genetically.
- Fourth reactor: corresponds to soil and surface and ground water, where the bacteria originated from previous reactors can blend or neutralize environmental organisms.

As Baquero et al. (2008) state, the possibility of reducing the evolution of antibiotic resistance depends on the ability of humans to control the flow of active antimicrobial agents, bacterial clones biological information along these genetic reactors.

The third reactor, corresponding to the wastewater treatment plants is, at present, the subject of studies by researchers seeking to understand where antibiotics go, as well as bacteria and resistant genes (Clara et al., 2005; Gómez et al., 2007; Jones et al., 2007; Joss et al., 2005). However, the number of studies focusing exclusively on the removal of ARB in wastewater treatment systems is still limited (Michael et al., 2013).

2.5. Wastewater Treatment Plants

The wastewater treatment plants (WWTP) are facilities that promote the cleanliness of the wastewater, through the removal of pollutants coming from sewage, in order to reduce the level of suspended solids and organic and inorganic content, allowing the reuse of water (Horan, 1990). WWTP are certainly the most appropriate fate of polluted waters, contributing for the protection of public health and the preservation of water resources, as they avoid their contamination (Gray, 2010).

According to Sonune and Ghate (2004), a conventional wastewater treatment consists in the combination of physical, chemical and biological processes and operations to remove solids, organic matter and sometimes nutrients from waters. Generally, the treatment of wastewaters proceeds in four steps: (i) pre-treatment; (ii) primary treatment; (iii) secondary treatment; and (iv) tertiary treatment.

2.5.1. Pre-treatment

Pre-treatment is essentially a physical process that aims reducing the solid content of the wastewater (Horan, 1990). When the wastewater arrives at WWTP, these residual waters contain a significant amount of floating material (wood, rags, paper and fecal material), as well as heavier solids, such as grit, and suspended solids (Horan, 1990; Sonune and Ghate, 2004). Pre-treatment

prepares wastewater to the following treatments, reducing part of the organic and inorganic matter in order to prevent damage of the mechanical equipment (Horan, 1990; Vesilind, 2003). This treatment includes normally: homogenization, to obtain a liquid flow with characteristics approximately constant; pH neutralization; flotation to oils, grass and suspended solids removal (Vesilind, 2003).

2.5.2. Primary treatment

Wastewaters which were subject to preliminary treatment have already much of the solid and floating material removed. However, they still contain high concentrations of suspended particles with a size ranging between 0.05 - 10 nm, which are known as settleable solids (Horan, 1990). It is not important that these settleable solids are removed prior to the biological treatment, and in fact many biological operating units are designed to operate without a primary settling. However, a sedimentation tank may be able to remove 25-50 % of biochemical oxygen demand (BOD), 50-70 % of suspended solids, and 65 % of fat and oil (Sonune and Ghate, 2004). The reduction of the BOD loading allows the use of smaller reactors in the following step, with low energy consumption and it also results in the production of less sludge, thus enabling smaller secondary sedimentation tanks (Horan, 1990; Vesilind, 2003). There are three types of sedimentation tanks which are used in accordance with the flow: vertical flow tanks, tanks and upward flow radial flow tanks (Horan, 1990).

2.5.3. Secondary treatment

The second phase, known as secondary treatment, typically depends on biological processes to remove organic matter (approximately 90%) and/or on nutrients in aerobic, anoxic or anaerobic systems (Michael et al., 2013).

The major biological processes for wastewater treatment can be divided into two categories: fixed biomass processes and suspended biomass processes (Horan, 1990; Vesilind, 2003). The fixed biomass process (such as trickling-bed) is in the oldest form of wastewater treatment (Horan, 1990). The growth of the microorganisms, responsible for the conversion of organic matter and nutrients, occurs on the surface of a stone or plastic material, and during the passage of the waste water, its purification occurs by water infiltration into the soil (Horan, 1990; Vesilind, 2003).

In the process of suspended biomass, predominantly aerobic, high concentrations of microorganisms are obtained through the recycling of biological solids. These microorganisms convert the organic constituents and inorganic fractions into new cells and by-products can be

removed from the system by gas extraction, sedimentation or other physical means (Vesilind, 2003).

Within the biomass suspended processes used to treat wastewater, the most widely used is the activated sludge process. This process consists of a system of microorganism growth in suspension where they are provided organic matter and oxygen. The microorganisms grow into flakes, which are responsible for the transformation of organic matter into new microbial biomass, carbon dioxide and water (Horan, 1990).

The activated sludge system is colonized by a number of organisms, including bacteria (main decomposers), fungi, protozoa and rotifers. The microbial population is dominated by heterotrophic organisms, such as bacteria, fungi and protozoa, some of which require biodegradable organic matter to acquire energy and synthesize new cells. Autotrophic bacteria, which are found in varying but usually low concentrations, have the ability to use inorganic matter to gain energy as well as biomass. Finally, protozoa and rotifers are only 5 % and they are grazers or predators that feed on other bacteria or protozoa and rotifers (Vesilind, 2003).

2.5.4. Tertiary treatment

After the biological treatment, comes the third phase of the process: the tertiary treatment. The tertiary treatment may be defined as any process of treatment in which unit operations are added to the flow regime after conventional secondary treatment (Sonune and Ghate, 2004). In this step, there is the disinfection of treated wastewater and the removal of some nutrients, such as nitrogen and phosphorus that were not removed by biological processes, which contribute to eutrophication of receiving waters (Batt et al., 2007; Sonune and Ghate, 2004). In this last phase it may also be used filtration to remove suspended solids and colloidal or chemical oxidation to remove contaminants (Sonune and Ghate, 2004).

2.6. Wastewater treatment plants as hotspot for development and dissemination of antibiotic resistant bacteria and genes.

According to Sim et al. (2010), WWTP are considered as one of the most important sources of antibiotics in the aquatic environment, because they were primarily made to remove classical contaminants (solids, nutrients and organic matter) and not emerging pollutants, such as pharmaceuticals (Clara et al., 2005; Nakada et al., 2007). Thus, according to various studies, wastewater treatment plants consisting of a variable mixture of bacteria, nutrients, and

antimicrobial agents in sub-inhibitory concentrations, become favourable locations for the proliferation of resistant bacteria, which in turn can transfer resistant genes to susceptible bacteria (Kruse and Sørum, 1994; Lindberg et al., 2004; Mach and Grimes, 1982; Poté et al., 2003).

Several studies have shown that the prevalence of ARB in the wastewater can vary significantly depending on the plant (the initial characteristics of the sewage, the type of treatment and the plant operation, among others), the target bacterial population and the antimicrobial agent being studied, as well as the methods used to determine antibiotic resistance (Guardabassi et al., 2002). As reported by Gao et al. (2012), who investigated the relation between the number of ARB and ARG and the concentration of tetracyclines and sulfonamides located in a WWTP in Michigan, there was a significant decrease of ARB and ARGs after the treatment. In a study by Iwane et al. (2001), in samples taken before and after chlorination in a WWTP, it was reported the presence of tetracycline resistant bacteria approximately 8% and 6.7%, respectively. In the same study, samples were taken near the place of discharge of the WWTP and it was found that the percentage of bacteria resistant to tetracycline was similar.

Munir et al. (2011) concludes that the WWTP are potential sources of ARB and ARG. The study consisted of the analysis of concentrations of bacteria and resistant genes to tetracycline and sulfonamide throughout the various steps of 5 WWTP in Michigam. Munir and his colleagues showed that despite a significant reduction of ARG and ARB in the raw effluent after the treatment, a final effluent with high concentrations of ARB and ARG was still discharged. Goñi-Urriza et al. (2000) evaluated the impact of a WWTP discharges into the environment by monitoring populations of ARB in the river Arga in Spain. Through the antibiotic susceptibility testing, it was found that the resistance of 21 out of 22 antibiotics tested was significantly increased among strains of Enterobacteriaceae and Aeromonas spp. collected at the local wastewater discharge.

In Portugal, Martins da Costa et al., (2006) conducted a study in 14 municipal WWTP to investigate the antimicrobial resistance of enterococci in the raw effluent, treated and in the mud. They detected the presence of multidrug resistance in 49.4% of the 983 isolates and also a high resistance to rifampicin, tetracycline, erythromycin and nitrofurantoin antibiotics. Furthermore, it was concluded that despite the decrease of enterococci in the final effluent, the concentration of enterococci was still quite high and therefore considered disturbing. In this study, it is also concluded that the worst performances in the reduction of bacterial load were in ancient WWTP and on rainy days.

2.7. Mechanisms to fight the spread of antibiotic resistant bacteria using wastewater treatment plants

As previously stated, current WWTP are not prepared for the effective elimination of ARB and antibiotics. Thus, it is important, in the future, to invest on the optimization of the design of the WWTP and new removal treatments (Kim and Aga, 2007).

The disinfection of wastewater is currently considered as a process capable of inactivating the microorganisms and may, thus, provide an opportunity to restrict the release of ARB and ARG to the environment (Bouki et al., 2013; Rizzo et al., 2013b). Thus, disinfection is regarded as a way to reduce the risk of the spread of pathogenic resistant micro-organisms to antibiotics. Thus, at some WWTP, the effluent is disinfected before being released to the environment through chlorination or ultraviolet (UV) radiation (Michael et al., 2013).

Chlorine is a widely used disinfectant due to its strong disinfection capability, easiness of application and low cost (Anastasi et al., 2013; Ma et al., 2013). Chlorine has several purposes, among them, the oxidation of the cells, the alteration of cellular permeability, the inhibition of enzymatic activity and the damage to DNA and RNA (USEPA, 1999). This mechanism of disinfection is dependent on several factors: the microorganism, the characteristics of the wastewater and the dose of chlorine (USEPA, 1999). However, chlorine is a strong oxidant which converts organic matter dissolved in water in products which have proved to be cytotoxic and genotoxic (Hijnen et al., 2006; Zhang et al., 2012).

Disinfection of sewage waters by UV radiation is considered as an alternative to chemical disinfection since it does not produce toxic products (Hijnen et al., 2006). Thus, in order to balance public safety and environmental protection with an effective disinfection, many WWTP replaced the chemical disinfection, such as chlorination, by a UV technology (Bouki et al., 2013). The UV radiation can damage the DNA, resulting in the inhibition of cell replication and, in the case of lethal dosages, the loss of reproducibility (Rizzo et al., 2013b). The effectiveness of the UV disinfection system depends on the characteristics of the wastewater, the UV radiation intensity, the time in which the microorganisms are exposed to radiation and the configuration of the reactor (Rizzo et al., 2013b). Although disinfection with chlorine or by UV radiation can be used as weapons against ARB and ARG, studies that report their removal are few and contradictory (Bouki et al., 2013).

In the study by Huang et al. (2011), there was an increase in the proportion of various ARB after chlorination especially by chloramphenicol resistant bacteria and also reactivation of some ARB when subjected to a low dosage of chlorination. Gao et al. (2012) verified there was no apparent decrease in the concentration of tetracycline and sulfonamide resistant genes by disinfection with chlorine. The study by Li et al. (2013), which had the purpose to investigate the inactivation, reactivation and also the regrowth of pathogenic bacteria in waters disinfected with chlorine, showed that despite a reduction in the rate of reactivation and re-growth of bacteria, there was a potential risk of prevalence and bacterial re-growth. Furthermore, it was shown that disinfection with chlorine resulted in a selection of chlorine-resistant pathogenic bacteria.

In the case of UV disinfection, Conner-Kerr et al., (1998) reported an elimination of 99.9% of methicillin-resistant strain of *Staphylococcus aureus* or vancomycin-resistant *Enterococcus faecalis* in vitro at a fluence of 77 mJ/cm-2. In studies by Guo et al., (2013), there is an effective reduction of the ARB in the final effluents after being submitted at a fluence of 5 mJ/cm^{-2}. However, in both studies, it was found that the UV disinfection may have selectivity for certain ARB, since there were increases and decreases of the proportion of certain ARB strains. Already in the study by Munir et al. (2011), it was found that there were no significant differences in the concentration of ARB and ARG before and after disinfection. The disinfection methods currently used are not fully effective against the spread of ARB. Thus, further studies are necessary for the application of disinfection methods effective for a complete inactivation/removal of ARB in WWTP (Bouki et al., 2013; Rizzo et al., 2013b).

Another way to reduce the spread of ARB, as well as preventing environmental changes, is the removal and transformation of antibiotics in WWTP through different processes.

According to Michael et al. (2013), the biological treatment does not remove completely the antibiotic in the treatment of wastewater and therefore alternative methods of treatment are considered as necessary to eliminate these compounds in the effluents. These processes can be biotic, i.e. biodegradation by bacteria or fungi, or abiotic, such as sorption, hydrolysis, photolysis, oxidation and reduction. Improvements of WWTP, as well as the use of technology, are considered as a possible optimization of biological treatment in what the removal of antibiotics is concerned.

The deployment of WWTP in hospitals is another way to fight the spread of pathogenic and antibiotic resistant microorganisms, since the hospital effluents are discharged in WWTP without any pre-treatment (Azar et al., 2010). Wastewaters generated in hospitals come from all medical activities (like surgery, first aid, etc.) and non-medical (such as kitchen and laundry) (Azar et al.,

2010). Besides, hospital waters have harmful pollutants such as biodegradable organic matter (protein, carbohydrate), medicinal residues and laboratory chemicals (antibiotics, phenol) and pathogenic organisms (bacteria, viruses) (Emmanuel et al., 2005; Richardson and Bowron, 1985).

The first fully-dedicated hospital wastewater treatment plant in Europe started operating in July 2011, in Marien hospital, in Gelsenkirchen, Germany. It was the first wastewater plant to focus on the elimination of micro-pollutants, as well as the purification of wastewater coming from hospital activities (MICRODYN-NADIR, 2012). In this WWTP, there is a large variety of treatment processes, such as ultrafiltration, ozonisation and activated carbon filtration.

2.8. Relevant microorganisms in hospitals that enter in the environment

As previously mentioned, the presence of bacteria in the environment has been concern to the population. However, most alarming is the presence of certain bacteria species known as "ESKAPE" pathogens in hospital and care unit environments. The "ESKAPE" pathogens group is constituted by following bacteria: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae, Acinetobacter baumanni*, *Pseudomonas aeruginosa*, and *Enterobacter* species (Rice, 2008). These microorganisms are capable of escape the effects of antimicrobial drugs and they currently cause the majority of hospital infections (Alicia I. Hidron et al., 2008; Rice, 2008). Of ESKAPE group, *P. aeruginosa* and *S. aureus* were selected for the present study because they are common microorganisms in the sewage, probably due to the entrance of hospital and care unit sewages in the urban WWTP.

2.2.1. *Pseudomonas aeruginosa*

P. aeruginosa is ubiquitous in nature and therefore it is possible to find in water, soil, plant, animals and man (Barnes et al., 2014; Giamarellou, 2000). Gessard was the first to isolate *P. aeruginosa* in 1822, but only in 1890 it was acknowledged as a pathogen by Charrin (Bodey et al., 1983)

P. aeruginosa is a bacterium and belongs to the family Pseudomonadaceae. It is a non-sporing gram-negative, rod shaped, and measures 0.5 to 0.8 μm by 1.5 to 3.0 μm (Iglewski, 1996). This bacterium has one polar flagellum that confers motility (Lovewell et al., 2011) The organism is an aerobe but can grow anaerobically if nitrate, used as electron acceptor, is present (Arai, 2011; Filiatrault et al., 2006). Several variants *P. aeruginosa* can be found with a range of colony morphologies including the classical mucoid phenotype (Foweraker et al., 2005).

P. aeruginosa can use many environmental compounds as carbon and energy sources (Williams and Worsey, 1976). Its optimum growth temperature is 37°C and no growth occurs at 5 °C (Iglewski, 1996; Palleroni, 2001). However, *P. aeruginosa* is able to grow at temperatures as high as 42°C. This characteristic allows to distinguish *P. aeruginosa* from other Pseudomonas species, such as *Pseudomonas putida* and *Pseudomonas fluorescens* (d'Agata, 2014; Iglewski, 1996; Oberhofer, 1979; Palleroni, 2001).

The identification of *P. aeruginosa* is relatively easy to make due to its ability to produce a variety of pigments (Norberto, 2008). The most encountered pigments are pyocyanin and fluorescein. Pyocyanin is blue/green pigment that is specifically produced by this species. Fluorescein is a yellow pigment and fluoresces under ultraviolet light, but it is produced by many other Pseudomonas species too. A few strains can also produce a brown (pyomelanin) or a red (pyorubin) pigment. In some cases atypical strains are non-pigmented, normally corresponding to environmental strains of *P. aeruginosa*. All strains of this species are characterized for being catalase and oxidase positive (Norberto, 2008; Yabuuchi and Ohyama, 1972).

The ability of *P. aeruginosa* to survive on minimal nutritional requirements and to tolerate a variety of physical conditions allows colonization in different sites, with a preference for moist environments (Lister et al., 2009; Schwartz et al., 2006). In the hospital, it is possible to isolate *P. aeruginosa* from a variety of sources, like respiratory therapy equipment, antiseptics, soap, medicines, and physiotherapy and hydrotherapy pools (Giamarellou, 2002; Pollack, 1995). This bacterium can be too found in community reservoirs like swimming pools, whirlpools, contact lens solution, home humidifiers, taps, sinks, drains, toilets, showers, vegetables, soil and rhizosphere (Halabi et al., 2001; Harris et al., 1984; Lister et al., 2009). They can even grow in distilled water, thus explaining their constant presence in the environment (Favero et al., 1971; Mena and Gerba, 2009).

They have an extensive impact at various levels, such as food spoilage and degradation of petroleum products and materials. It is also an important pathogen with a remarkable range of hosts, including mammals, insects, worms, amoeba, fungi, and other bacteria (Mena and Gerba, 2009; Siryaporn et al., 2014). Of all pseudomonads, *P. aeruginosa* is the most important opportunist pathogen either for healthy or compromised humans (Mena and Gerba, 2009). In case of healthy individuals, *P. aeruginosa* is responsible for ear, eye and skins infections acquired from contaminated water (Mena and Gerba, 2009). These infections are caused due to extended water-

to-skin contact in damaged tissues and, in the case of eye infections, it occurs due to minor tiny scratches caused by the wearing of contact lenses (Mena and Gerba, 2009).

Most infections caused by *P. aeruginosa* occur in hospital environment where the colonization rates may exceed 50% during hospitalization (Bodey et al., 1983; Pollack, 1995). This organism is responsible for approximately 10 % of nosocomial infections, and is therefore considered the major nosocomial pathogen (Dembry et al. 1998). Nosocomial infections caused by *P. aeruginosa* include pneumonia, urinary tract infection, surgical site infections, and septicemia (Spencer, 1996). Patients at the greatest risk of infection are burned patients, cystic fibrosis patients, individuals infected with HIV, elderly persons, premature infants and patients which had a disruption in the normal microbial flora as a result of antimicrobial therapy (Botzenhart and Döring, 1993; Mena and Gerba, 2009; Takesue et al., 2002). Infections caused by *P. aeruginosa* are not only common, but they are also associated with high morbidity and mortality (Driscoll et al., 2007). These infections are very hard to eradicate and a major cause for therapeutic failure is due to the high number of virulence factors, as well as multiple antibacterial resistance mechanisms (Driscoll et al., 2007; Hancock and Speert, 2000; Li et al., 1994).

P. aeruginosa is intrinsically resistant to several antibiotics such as β-lactams, macrolides, tretracyclines and most fluoroquinolones (Driscoll et al., 2007). This resistance may be due to the low permeability of the outer-membrane, the constitutive expression of various efflux pumps, and the production of antibiotic-inactivating enzymes (Hancock, 1998). Although *P. aeruginosa* is not intrinsically resistant to others antibiotics, like carbapenems or polymyxins, it is able to develope resistance or to acquire new mechanisms of resistance (Mesaros et al., 2007). According to Vaisvila et al., (2001) this could be due to the large size and the versatility of its genome, and to its ubiquitous in aquatic environments, which could be also a reservoir for bacteria carrying other resistance genes. So, the capacity of *P. aeruginosa* to rapidly mutate and adapt to antibacterial threats can help to develop a multidrug-resistant phenotype and, in the worst case, a superbug (resistant to practically all antimicrobial drugs available) (Breidenstein et al., 2011; Lister et al., 2009).

The pathogenicity of this organism is due to an arsenal of virulence factors. Among them are cell-associated factors (such as lipopolysaccharide, flagella and pili, the exopolysaccharide alginate) and secreted factors (such as toxins, elastase and other proteases, phospholipases, pyocyanin) (Gooderham and Hancock, 2009; Van Delden and Iglewski, 1998). Quorum-sensing, type III secretion (pathway to inject virulence proteins into the cytosol of their eukaryotic host cells) and

many other regulatory systems are important for virulence (Gooderham and Hancock, 2009; Mota et al., 2005). These bacteria attach to the host cell surface during the infection process and this surface act as a cue for *P. aeruginosa*, signaling the presence of a host. As previously mentioned surface attachment is a critical step that enables the creation of biofilms, which are important for pathogenesis (Siryaporn et al., 2014).

2.2.2. *Staphylococcus aureus*

S. aureus, a member of the *Staphylococcaceae* family, was discovered in 1880 by the surgeon Sir Alexander Ogston, in human pus (Foster and Geoghegan, 2015; Ogston, 1984). It is a Gram-negative, non-spore-forming and non-motile spherical organism, which presents 0.5 to 1.5 μm of size (Foster and Geoghegan, 2015; Mathema et al., 2009). *S. aureus* is a facultative anaerobic, capable to ferment a various sugars, such as mannitol, sucrose and maltose, producing acid but no gas (Charlier et al., 2009; Foster and Geoghegan, 2015). It is coagulase and catalase-positive, but oxidase-negative (Foster and Geoghegan, 2015; Sperber and Tatini, 1975) The production of coagulase by *S. aureus* allows to distinguish it from other staphylococcal species (Foster 1996).

The bacterium is capable to grow at a temperature range of 7–48 °C (optimum 37 °C) (Kadariya et al., 2014) *S. aureus* originates large, circular, convex, smooth, shiny and opaque colonies on nutrient agar and the majority produce a golden yellow pigment, which is not diffusible into the medium. However, to identify and isolate *S. aureus*, it is usually used mannitol salt agar (MSA), a selective and differential medium for *Staphylococcus* spp. As a result of mannitol fermentation, normally pink medium turns yellow as well as colonies of *S. aureus* (Engelkirk and Duben-Engelkirk, 2008).

S. aureus is a ubiquitous organism and its predominant reservoir is the human being (Richard et al., 2005; Shirtliff et al., 2002). Although the multiple body sites where it can be found, such as axillae, intestine and perineum, it is an excellent colonizer of the moist squamous epithelium of the anterior nares. In fact, this is the most frequent carriage site for *S. aureus* (Aly and Levit, 1987; Foster and Geoghegan, 2015; Williams, 1963). According to Foster (2004) and Klutmans et al., (1997), about 20% of the population always carries one type of strain (persistent carriers); 60% carries different strains which frequently change (intermittent carriers); and only 20% do not carry this bacterium, (noncarriers). Carriage of *S. aureus* appears to play an important role in the epidemiology and pathogenesis of infections (Kluytmans et al., 1997). Carriers are presumed to be an important source spreading among individuals. Normally, the primary mode of transmission is by direct contact, skin-to-skin exchange through colonized or infected individuals (Lowy, 1998;

Miller and Diep, 2008). The contact with contaminated objects and surfaces constitute another form of transmission or even of infection, when it comes to medical equipment (Muto et al., 2003).

This organism has the ability to cause a high variety of diseases, from benign skin infections to necrotizing pneumonia and infective endocarditis (Lowy, 1998). *S. aureus* is known for causing many skin-related infections such as atopic dermatitis, carbuncles, cellulitis, follicles and furuncles (Archer, 1998; Foster and Geoghegan, 2015; Otto, 2010). It also provokes serious infections in persons debilitated by chronic illness, traumatic injury, burns or immnosuppressions (Archer, 1998; Foster 1996; van Kessel et al., 2003). In these cases the infections caused include pneumonia, deep abscesses, osteomyelitis, endocarditis, phlebitis, mastitis, meningitis, bacteremia and septic shock (Archer, 1998; Foster 1996; Otto, 2010). This pathogen is a major cause of infections associated with indwelling devices like as joint prostheses, cardiovascular devices, artificial heart valves, intravascular and intraperitoneal catheters (Foster 1996; van Kessel et al., 2003). Such as *P. aeruginosa*, *S. aureus* is an opportunistic pathogen that contributes significantly to morbidity and mortality (Sollid et al., 2014). Usually their infections are associated with hospitalized patients rather than healthy individuals in the community (Foster 1996).

Outside the hospital environment, beaches have been suggested as a potential source of community-acquired *S. aureus* infection such as skin, eye and ear infections namely among bathers (Calderon et al., 1991; Charoenca and S. Fujioka, 1995; Gabutti et al., 2000). Despite the source of *S. aureus* on the beach be associated with human activity, this microorganism has already been found in stormwater, coastal streams and wastewater which may be another source of *S. aureus* dissemination on the environment (Goodwin et al., 2012; Selvakumar and Borst, 2006; Viau et al., 2011). *S. aureus* is also known to be a cause of food poisoning through the release of enterotoxins (Baird and Lee, 1995; Foster and Geoghegan, 2015).

S. aureus is naturally susceptible to various antibiotics; however due to the intense selective pressure, it already acquired resistance to virtually every class of known antibiotics such as penicillin, methicillin, or vancomycin (Chambers, 2001). Since the beginning of the antibiotic era, *S. aureus* has responded to the introduction of new drugs by rapidly acquiring resistance through a variety of genetic mechanisms (Foster 1996; Schito, 2006). Because of that, the situation of antibiotic resistance in this organism is considered alarming and moreover this resistance is moving from the hospital to the community (Schito, 2006). According to Otto (2010), is the combination of antibiotic resistance with high virulence that makes this bacterium a dangerous pathogen.

S. aureus can express a wide number of virulence factors that allow it to adhere to surfaces, invade or avoid the immune system, and cause harmful toxic effects to the host (Foster, 2004; Holmes et al., 2005; Lowy, 1998). This pathogen expresses virulence factors such as: surface proteins that promote colonization of host tissues (proteins that bind to fibrinogen, fibronectin, collagen and laminin); factors that probably inhibit phagocytosis (capsule, immunoglobulin binding protein A); and toxins that damage host tissues (α-toxin, β-toxin, δ-toxin, γ-toxin and leukocidin) and cause disease symptoms (enterotoxins and toxic shock syndrome toxin) (Archer, 1998; Foster 1996; Foster and Geoghegan, 2015). Biofilm formation is a virulence mechanism, which allows bacteria to persist and resist the host defenses or the antibiotics, as well as small colony variants (SCV) of *S. aureus* which facilitate persistent and recurrent infections (Foster, 2005; von Eiff et al., 2006).

CHAPTER III| MATERIALS AND METHODS

The procedures that were performed during the experimental work are described in this chapter. Briefly, samples were collected throughout the year from three different locations of the wastewater treatment process. These three samples were treated to analyse the effectiveness of ultraviolet radiation in the reduction of bacteria, especially *P. aeruginosa* and *S. aureus*. In order to analyse some virulence factors of these two pathogens bacteria present in the samples, some colonies were isolated and numbered. After that, the ability of different colonies of each species to form biofilms was analysed and the susceptibility to selected antibiotics was also assessed. The flow diagram of the experimental work is shown in Figure 2.

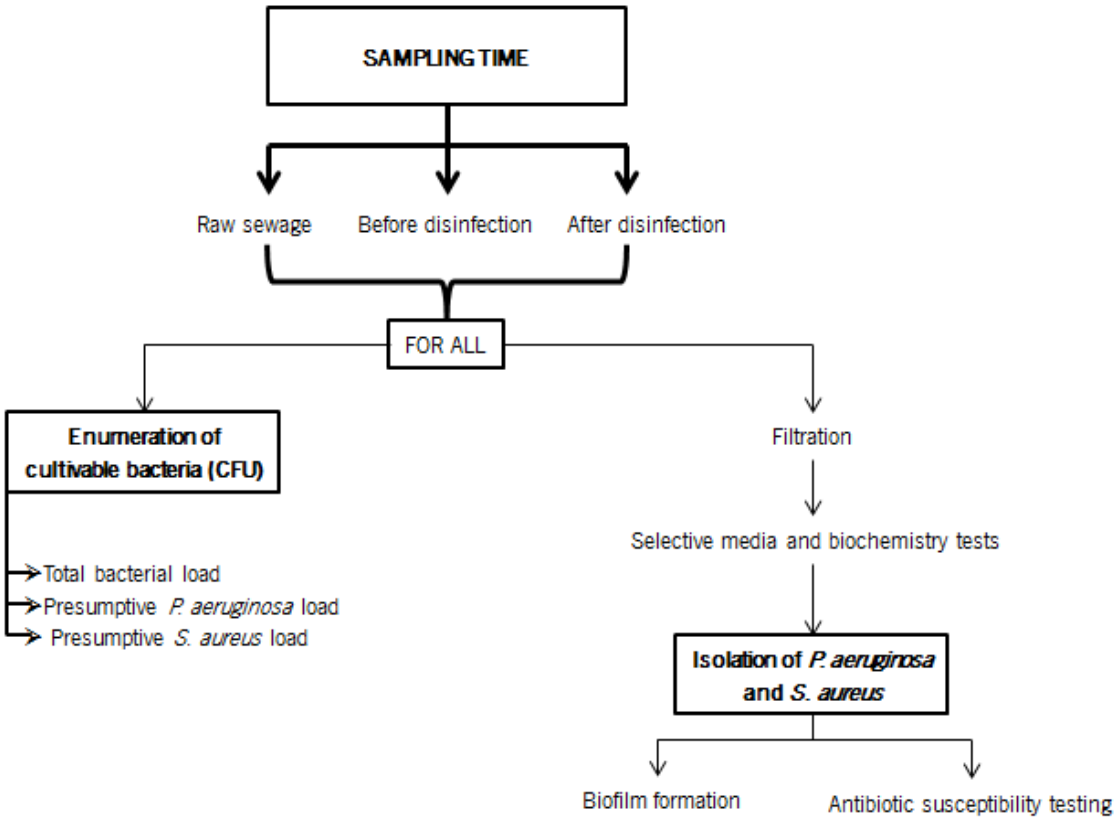

Figure 2| Flow diagram of the work experimental performed during the year.

3.1. Sampling

To characterize the microbial population, the wastewater samples were collected in one WWTP during a period of four months (March to June). In order to compare the amount and variety of total microorganisms, principally *P. aeruginosa* and *S. aureus*, during the different stages of waste water treatment, samples were collected in three different locations: at the entrance of the sewage

treatment plant, at the exit before and after the disinfection stage. All samples were transported inside appropriate bottles and immediately analysed.

3.2. Enumeration of cultivable bacteria

In order to verify the general effectiveness of disinfection by UV in the WWTP, the number of viable and cultivable cells was determined through the counting technique of Colony Forming Units (CFU), which is based on the principle that each present viable and cultivable cell will multiply itself and form a colony of cells.

For each collected sample successive dilutions (up to 10^{-8}) were performed with sterile distilled water. In each previously labelled Tryptic Soy Agar (TSB; 30 g/L, Liofilchem, Italy, supplemented with Agar;12.5 g/L, Liofilchem, Italy) petri dish 3 drops of 10 µL of each performed dilution (included the initial sample) were placed. The plate was then tilted so the drops could drain.

This procedure was repeated for the counting of target bacteria (*P. aeruginosa* and *S. aureus*) using the respective selective media and, in the case of plates containing Cetrimide Agar (CA; 45 g/mL,Merck, Germany, supplemented with glicerol), this was performed in duplicate in order to undergo 37 °C and 42 °C to differentiate *P. aeruginosa* from other species of *Pseudomonas* (Khan and Cerniglia, 1994; Reyes et al., 1981). Mannitol Salt Agar (MSA; 111 g/L, Liofilchem, Italy) was the selective media used to *S. aureus*. Thus, the plates were incubated at 37 °C (TSA, MSA and CA) and 42 °C (CA) for 18h-24h. After incubation, plates containing the appropriate dilution, i.e., that resulted in 30 to 300 colonies, were chosen to perform the counting and recording of data (adapted from Gusbeth et al., (2009).

3.3. Isolation of bacteria

3.3.1. Filtration

The isolation of the target bacteria that would be subsequently used for the determination of virulence and antibiotic-resistance, was performed by a membrane filtration method (adapted from Rizzo et al., (2013a). After the samples homogenization, volumes of 25 mL and 50 mL were filtered through 0.22 µm membrane filters (Advantec, Japan). Some samples had to be diluted before being submitted to filtration. This procedure was performed in duplicate and the membranes were placed in the plates with CA and MSA. Thereafter the plates were placed into the incubator at 37 °C and they were incubated overnight. The next day the membranes were removed

with the aid of sterile loops to enable the subsequent manipulation of the colonies aiming at the isolation of the target bacteria (procedure described in 3.3.2).

Simultaneously, an extra filtration was performed and this membrane was placed in TSA to proceed to the cryopreservation (procedure described in 3.3.3) of the biomass present in the initial samples. The described procedure was performed, at each sampling time, for the three locations selected for the collection of samples: entrance, exit previous to the disinfection and exit after the disinfection.

3.3.2. Selective media and biochemistry tests

In order to identify and isolate several colonies of *P. aeruginosa* and *S. aureus*, conventional methods of microbiology were used. Conventional methods are based on phenotypic characterization by inspection of the morphology, the physiology and the biochemical reactions carried out by bacteria. However, confirmation of the identifications by these phenotypic methods is not 100% guaranteed because evaluation of results can be subjective (Gulluce et al., 2014). So it would be valuable to complement this evaluation with molecular methods. Those were not possible to accomplish yet. Nevertheless, in order to guarantee the diversity of the samples, three possibly different colonies of each sample was isolated in each of the sampling site and time.

3.3.2.1. Isolation of *P. aeruginosa*

Due to the difficulties presented in the identification and isolation of presumptive colonies of *P. aeruginosa* by conventional methods, it was necessary to change them. Thus, the follow methods described correspond to adapted and optimized methods, in the scope of the present work.

After removal of the membranes from CA petri dish, four plates of the same medium for each sample were subcultured in order to obtain isolated colonies. These plates were incubated overnight at 42 °C.

The next day 8-10 colonies were selected from each sample and characterized according to morphological features (diameter, shape, surface, edge and colour). After characterization, the selected colonies were removed with a sterile loop, picked into previously identified CA petri dishes, and incubated overnight at 42 °C. Later a new isolation was made in order to be sure that a pure colony would be obtained. After the second isolation, the colonies were subcultured to TSA petri dish and incubated overnight at 37 °C. This subculture had the purpose of obtaining fresh cultures of all isolated colonies for the oxidase test to be performed the next day (Mcavoy et al.,

1989).Oxidase Test Stick (Merck, Germany) was used to perform the oxidase test. This is a rapid test for determining the oxydasecytochrome enzyme. The procedure was performed according to the manufacturer instructions. According to the instruction sheet, reactions are considered positive if there is a colour change, i.e. the appearance of blue-purple colour (within 10 seconds) and negative reaction when no colour change occurred. Two control strains were used: *P. aeruginosa* ATCC 27853 as a positive control and S. aureus ATCC 29213 as negative control.

3.3.2.2. Isolation *of S. aureus*

Isolation of S. *aureus* colonies was similar to the isolation of *P. aeruginosa*, with some modifications. The medium used was MSA, a selective and differential medium, and inoculation occurred at 37 °C. The biochemical tests chosen for a presumptive identification of *S. aureus* were the following (Harastani et al., 2014):

Catalase test – Some drops (4-5) of hydrogen peroxide were added directly to overnight grown colonies on TSA plates. If vigorous bubbling occur (within 10 seconds), it indicates the presence of catalase and the result is deemed positive. If no bubbling occurs, the result is negative.

Coagulase test – to performe this test, the Coagulase Test (tubes) by Liofilchem (Italy) was used. This test was designed for the detection of coagulase enzyme produced by *S. aureus*. The procedure was performed according to the instruction sheet of the product. Two control strains were used: *S. aureus* ATCC 29213 as a positive control and *S. epidermidis* as a negative control. The interpretation of coagulase test was made according to Turner and Schwartz .A positive reaction occurs when any degree of clotting in the coagulase plasma occurs (Figure 3).

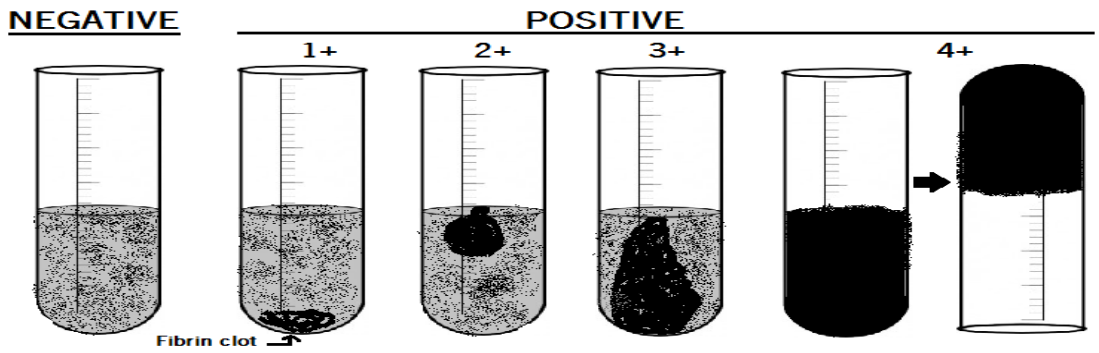

Figure 3| Interpretation of the coagulase test as proposed by Turner and Schwartz, where: Negative – no evidence of fibrin formation; 1+ Positive – small unorganized clots; 2+ Positive – small organized clot; 3+ Positive – large organized clot; 4+ Positive – entire content of tube coagulates and is not displaced when tube is inverted (Adapted from Turner and Schwartz (1958)).

After analysing the results of the tests performed, colonies of presumptive *S. aureus* were chosen and they were proceeded to cryopreservation (procedure described in 3.4.3). From all initially isolated colonies, those which had positive results in the majority of tests performed were chosen and they proceeded to cryopreservation (procedure described in 3.4.3).

3.3.3. Cryopreservation

Cryopreservation was performed with two purposes. The first was to preserve the biomass of initial samples present infiltration membranes placed in TSA. Thus, if something went wrong it would be possible to recover the existing microorganisms in the sample. The second aim of cryopreservation was to preserve different microorganisms isolated that, subsequently, would be tested to characterize some virulence factors. In the case of plates containing filters, after an overnight incubation at 37° C, portions of the biomass were removed with a sterile loop. These portions were placed into sterile cryovials containing 1.5 mL Tryptic Soy Broth (TSB; 30 g/L, Liofilchem) with 20% glycerol previously autoclaved (Anastasi et al., 2013). Subsequently, cryovials were placed to cryopreserve at -80° C. The same procedure was executed for cryopreservation of isolates from different samples. Anticipated subculturing of isolates was necessary in order to obtain fresh cultures for the cryopreservation.

3.4. Biofilm formation

The procedure for biofilm formation was performed in the same way both for control strains (*P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923) and bacteria isolates. The first step in biofilm formation was the preparation of the inoculum.

3.4.1. Preparation of the inoculum

The colony was removed from TSA plate with fresh culture and placed in 20 mL of TSB in a sterile Erlenmeyer. It was then incubated for 18 h in an orbital shaker (120 rpm) at 37 °C. The next day 1.5 mL of suspension were transferred to an Eppendorf tube and placed in a centrifuge for 6 min at 9000 rpm. The supernatant was discarded and the pellet was suspended in 1.5 mL of TSB and homogenized on a vortex mixer. The absorbance was then measured at 670 nm in a microplate reader (Sunrise TM, Tecan) and by using the calibration curve, a volume of 20 mL was adjusted to a concentration of $1x10^7$ CFU/mL. Biofilm formation was performed in 96-well microplate flat bottom polystyrene sterile (Orange Scientific), in which 200 µL of suspension was

placed. Two microplates were used for each microorganism since two distinct techniques would be applied: crystal violet staining technique and CFU technique. Microplates were incubated for 24 h at 37 °C with an agitation of 12 rpm. The TSB medium was used as a negative control.

3.4.2. Crystal violet staining technique

The Crystal Violet method (CV method) was used to evaluative the quantity of biomass formed. This dye stains all the biofilm biomass. After 24h incubation, bacterial suspension was removed of one of the 96-well plate and discarded. The plate was washed twice with sterile distilled water and 200 µL of methanol were added in each well for 15 min. After 15 min, methanol was discarded and the sample was left to dry. Thereafter 200 µL of 1% CV was placed into each well for 5 min. The plate was washed twice and left to dry. Finally, 200 µL of 33% acetic acid were added to each well and the absorbance was measured at 570 nm.

3.4.3. Colony forming units technique

The viable and cultivable biofilm-cells were determined by CFU quantification. The bacterial suspension of the other 96-well plate, after 24 h incubation, was removed and discarded. The plate was washed twice with sterile distilled water and, after discarding the water, 200 µL of the same water were added to each well. Sonication followed for 6 minutes by using an ultrasonic cleaning bath (Sonicor). After the bath, the contents of five wells were transferred to an Eppendorf tube and this procedure was repeated three times. Decimal dilutions were performed to each Eppendorf tube until 10^{-7} dilution.10^{-1} to 10^{-7} dilutions were plated on TSA plates. The plates were incubated between 18 and 24h (depending on the bacteria) at 37 °C. Afterwards, the number of colony forming units was counted in each dilution and data was recorded.

3.5. Antibiotic Susceptibility Testing

Nowadays, antimicrobial resistance has a great importance in public health at the global level. Thus, Antimicrobial Susceptibility Testing (ATS) is performed in order to detect possible drug resistance in pathogenic microorganisms and also to ensure susceptibility to the drugs selected for treatment (Reller et al., 2009). AST can be performed by using a variety of techniques, such as broth microdilution, gradient diffusion and disk diffusion methods (Reller et al., 2009; Schwalbe et al., 2007). Only the disk diffusion method was used in this study. The disk diffusion susceptibility method is one of the oldest approaches to AST and is considered a very simple, practical and well-

standardized method (Matuschek et al., 2014). Thus, susceptibility of bacteria to antibiotics was verified through tests with different antibiotics carried out by modified Kirby-Bauer disk diffusion method. To execute this method, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) methodology was followed. The standardized procedure was based on the principles defined in the report of the EUCAST (EUCAST, 2014b). The following collection bacteria were used as controls: *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923.

3.5.1. Preparation of media

Mueller Hinton Agar (MHA; 36 g/ L, Liofilchem, Italy) was prepared according to the manufacturer's instructions. However it is necessary to emphasize that medium would have a level depth of 4 mm 0.5 mm (approximately 25 mL in a 90 mm circular plate). If plates were too shallow they would have had produced false positives, due to the tested antimicrobial compound diffusing farther than it should, and thus creating larger zones of inhibition. On the other hand, if the plates were poured to a depth bigger than 4 mm, it could result in false resistant results (EUCAST, 2014b).

3.5.2. Preparation of antibiotic stock solution

The antibiotics used were chosen according to EUCAST breakpoint table (EUCAST, 2014a). In order to get the same amount of antibiotic of the EUCAST disks (Table 1), a stock solution of each antibiotic was prepared to obtain the mass necessary in 10 µL (volume to put on disk). Solvents, diluents and storage conditions for antibiotics were followed according to the manufacturer's recommendations.

Table 1| Disk content (µg) presented in EUCAST breakpoint table (EUCAST, 2014a). The antibiotics used were: piperacillin (PIP); ciprofloxacin (CIP); gentamicin (GENT); amikacin (AMK); tobymicin (TOB); Tetracycline (TETRA); chloramphenicol (CHF); rifampicin (RIF).

Classes of antibiotics	Antibiotics	Disk content (µg)
Penicillins	PIP (Sigma)	30
Fluoroquinolones	CIP (Fluka)	5
Aminoglycosides	GENT (Sigma)	10
	AMK (Sigma)	30
	TOB (Sigma)	10
Tetracyclines	TETRA (Applichem)	30
Miscellaneous agents	CHF (Sigma)	30
	RIF (Sigma)	5

3.5.3. Preparation of the inoculum

According to the EUCAST method, McFarland standards that consisted in using direct colony suspension and adjusting the turbidity of this suspension to a 0.5 McFarland standard, approximately corresponding to 1-2x10^8 CFU/mL (EUCAST, 2014b), should have been used. However this method was not employed. A calibration curve was used to adjust bacterial suspension to a cellular concentration of 1x10^8 CFU/mL. The preparation of the inoculum was performed as described in 3.4.1. However, cellular concentration was adjusted to 1x10^8 CFU/mL.

3.5.4. Preparation of the disks

The preparation of the disks is made in advance in order to give the disks time to dry. Sterile disks were removed (Liofilchem, Italy) from the cartridge with the aid of sterile tweezers and they were placed on previously identified sterile glass Petri dishes. A different dish was used for each antibiotic. 10 µL of stock solution was added to each disk, which was allowed to dry in laminar flow cabinet (Guérin-Faublée et al., 1996).

3.5.5. Inoculation of agar plates

Sterile cotton swabs were dipped into the Falcon tube, where it was suspended. The excess fluid was removed by turning the swab against the inside of the container. Afterwards, the swab spread the inoculum evenly over the entire surface of a plate, by passing the cotton swab three times and by rotating the plate approximately 60 degrees each time (EUCAST, 2014b; Schwalbe et al., 2007).

3.5.6. Application of antimicrobial disks and incubation of plates

After the inoculation of the plates, the disks should be applied on the surface of agar within 15 min. If plates already inoculated are left at room temperature for a long time before application of the disks, the organism may begin to grow. This can lead to erroneous results since that can reduce the size of inhibition zones (EUCAST, 2014b).

Disks containing antibiotic were placed in the dish with the aid of sterile tweezers. The disks were placed carefully in order not to stay at the edge of the plate because that hampers the measurement of inhibition zones. The distance between the centres of disks should be more than 24 mm (EUCAST, 2014b). Plates were inverted and incubated with agar side up, at 35±1°C for 16-20 h.

3.5.7. Examination of plates after incubation

After removal of the samples from the incubator, they were examined. According to EUCAST, growth should be uniform across the plate to obtain uniformly circular inhibition zones in a confluent lawn of growth (EUCAST, 2014b).

3.5.8. Measurement of zones and interpretation of susceptibility

For measuring the inhibition zones it is needed to take into consideration the following parameters (EUCAST, 2014b):

- Placed plates from the back;
- Plate should be against a dark background illuminated with reflected light;
- Measure zone diameters with a ruler and should be measured to the nearest millimetre(figure 4);
- The diameter of the disk is 0.6 mm;

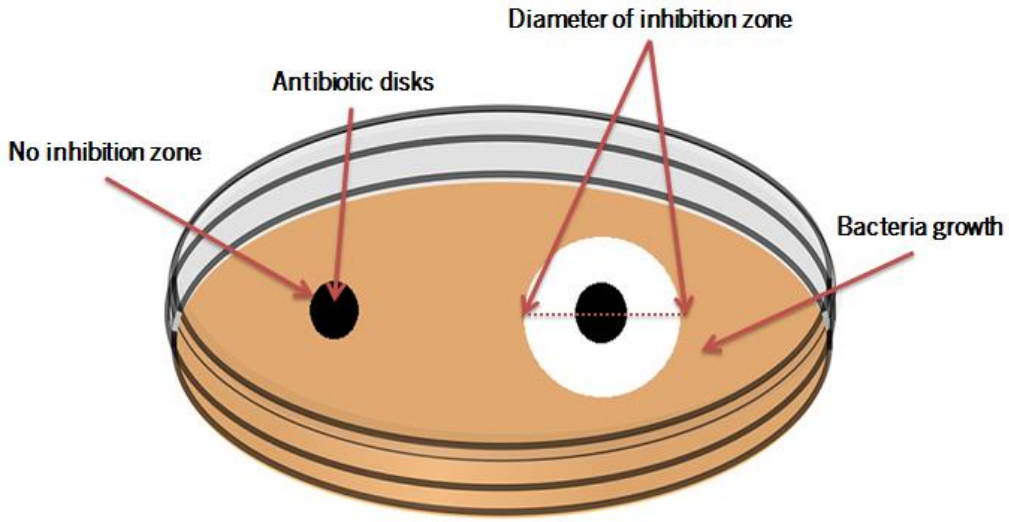

Figure 4| The antimicrobial susceptibility disk diffusion test: MHA plate with two antibiotic disks. One of disks have inhibition zone and other not shows inhibition zone.

- Check, firstly, if zone diameters for control strains are within acceptable ranges;
- Measured zone diameters are interpreted into categories of susceptibility according to the EUCAST Breakpoint Tables (table 2);

Table 2| Zone diameter breakpoint presented in EUCAST breakpoint table (EUCAST, 2014a) and concentrations of antibiotics tested. The antibiotics used were: piperacillin (PIP); ciprofloxacin (CIP); gentamicin (GENT); amikacin (AMK); tobymicin (TOB); tetracycline (TETRA); chloramphenicol (CHF); rifampicin (RIF).

		Zone diameter breakpoint (mm)			
		S. aureus		*P. aeruginosa*	
Classes of antibiotics	**Antibiotics**	S*≤	R*>	S*≤	R*>
Penicillins	PIP	-	-	18	18
Fluoroquinolones	CIP	20	20	25	22
Aminoglycosides	GENT	18	18	15	15
	AMK	18	16	18	15
	TOB	18	18	16	16
Tetracyclines	TETRA	22	19	-	-
Miscellaneous agents	CHF	18	18	-	-
	RIF	26	23	-	-

*S – susceptible and R – resistant.

- If it is not possible to measure the zone completely, it should be measured from the centre of the disk to its edge area and multiply by two, in order to determine the diameter;
- If colonies appear within the zone or if distinct colonies appear, those colonies should be subcultured, the purity checked and the test repeated, if necessary.

3.6. Data statistical analysis

Data analysis was performed in the statistical program Prism (GraphPad Prism 6 for Windows). Nonparametric, One-way ANOVA (Kruskal-Wallis) followed by Dunn's multiple comparison test were used for biofilm formation dates. In all the performed analyses results are considered statistically significant when $p < 0.05$.

CHAPTER IV| RESULTS AND DISCUSSION

This chapter is divided into four sections. The first section is about the efficiency of the treatment system in reducing the bacterial load, particularly *S. aureus* and *P. aeruginosa*, the latter considering the results obtained at the two referred temperatures (37 °C and 42 °C). In the second section, the characteristics of the chosen isolated colonies of *S. aureus* and *P. aeruginosa* (morphology and response to the biochemical tests) are presented. In the third section, the results of the resistance or susceptibility of the isolated colonies for different antibiotics, determined through the modified Kirby-Bauer disk diffusion method are shown. The fourth section presents the results of the biofilm formation experiments, namely the amount of biomass in the biofilm and biofilm cell quantification

4.1. Effect of wastewater treatment plant processes on reduction of bacterial load

Samples from three locations were collected with the aim of analysing the efficiency of the WWTP with a tertiary treatment disinfection system in reducing the total bacterial load and also *S. aureus* and *P. aeruginosa* load, used as indicators of the removal ability, present in the final effluent. The first location was at the entrance of the sewage treatment plant; the second at the exit before the disinfection stage and, finally, at the exit after disinfection. Four samples were collected between the months of March and June in order to observe a possible temporal variability. The results of the bacterial load occurring in the entrance and final effluents in the studied WWTP are illustrated in figures 5, 6 and 7.

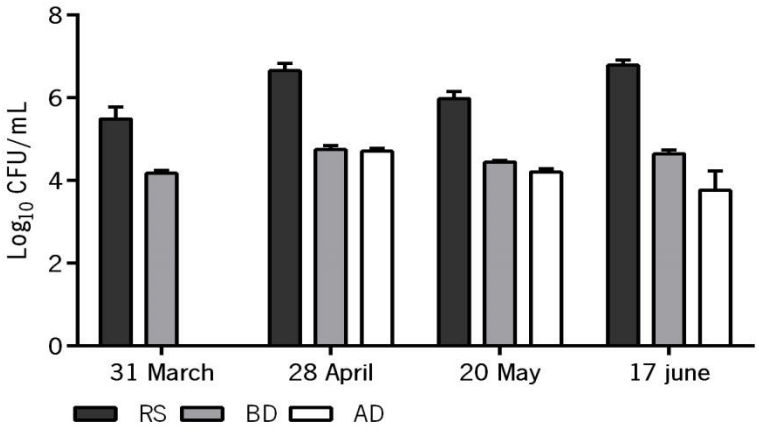

Figure 5| Total bacterial load (Log_{10} CFU/mL), of four samples, from three different locations: raw sewage (RS); before disinfection (BD); after disinfection (AD). Growth in TSA medium at 37 °C.

Figure 5 reveals an almost uniform behaviour between temporal samples. A reduction above 1 log unit of the total bacterial load is observed between raw sewage (RS) and before disinfection (BD) samples. The greatest reduction (approximately 2 log units) was observed in the sample from 17 June. In relation to before and after disinfection (BD and AD) samples, a significant reduction is not observed, with the exception of the sample from 31 March. In this sample, there was a total elimination of the bacterial load, which can be related to the eventual presence of some chemical compound or to a punctual low solid load that enable the UV action.

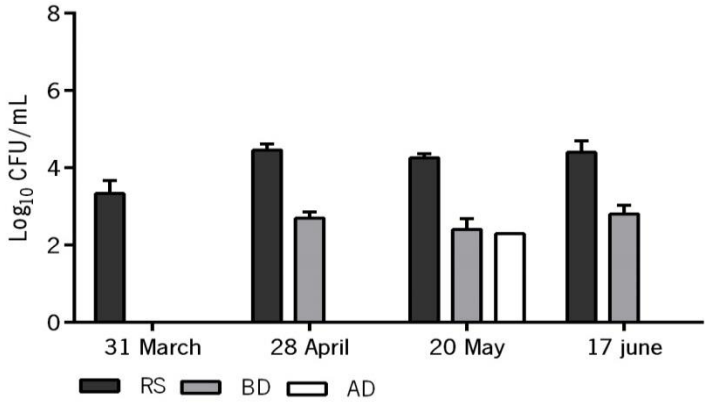

Figure 6| *S. aureus* bacterial load (Log_{10} CFU/mL) of four samples from three different locations: raw sewage (RS); before disinfection (BD); after disinfection (AD). Growth in MSA medium at 37 °C.

Figure 6 shows the variation of *S. aureus* bacterial load. In the sample from 31 March, *S. aureus* is only observed in the raw effluent. In the samples from 28 April and 17 June, however, *S. aureus* is present both in RS and BD. In both samples, the bacterial load in BD is half of that in RS. AD there is no bacterial load in what *S. aureus* is concerned. *S. aureus* is detected in the samples taken from the three locations of the WWTP in 20 May. The bacterial load is reduced to half from the RS to the BD, but the bacterial load in AD is practically the same as that in BD at this date.

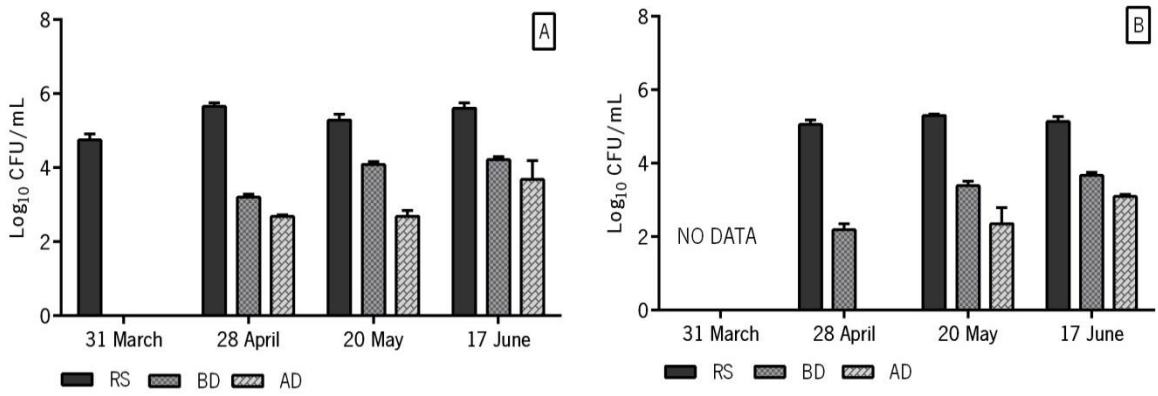

Figure 7| *P. aeruginosa* bacterial load (Log_{10} CFU/mL) of four samples taken in three different locations: raw sewage (RS); before disinfection (BD); after disinfection (AD). Growth in CA medium at 37 °C (A) and 42 °C (B).

P. aeruginosa bacterial load at 37 °C (A) and 42 °C (B) is shown in Figure 7. As in the previous cases, in the sample from 31 March at 37 °C, *P. aeruginosa* is only present in RS. No data was retrieved from this sample at 42°C. There is a bacterial load reduction either at 37 °C or at 42 °C (approximately 2.5 log units) from the RS to the BD in the sample from 28 April. The reduction obtained from BD to the AD is different between the tested temperatures. At 37 °C there is almost no reduction, while at 42 °C the presence of *P. aeruginosa* was not observed in AD. In the samples from 20 May and 17 June there is a reduction of 1.5 log units, approximately, from the RS to the BD, at both temperatures. There is a 1 log reduction from BD to AD in the sample taken at 20 May. There is no change in the sample from 17 June.

The data suggests that despite significant reductions from the RS to BD, the same does not happen in the AD samples. In general the disinfection did not reduce the total bacterial load, which remained high, with the exception of sample from 31 March, where there was no presence of any bacteria on AD. This leads to the conclusion that disinfection is not effective at eliminating the bacterial load. This becomes more concerning when the same occurs particularly to *P. aeruginosa*, since this is a pathogenic bacterium.

This lack of efficiency by the WWTP may be due to several factors. According to several studies, the presence of suspended particles in wastewater can increase microbial survival by shielding microorganisms from UV irradiation (Hassen et al., 2000; Macauley et al., 2006; Qualls et al., 1985). These particles are not penetrated by radiation, and thus they may offer protection to the bacteria, affecting directly the effectiveness of the UV disinfection suggested that the used UV-dose is not sufficient to inactivate *P. aeruginosa*, thus explaining the (Macauley et al., 2006). The application of low UV-doses can be another factor that may explain the high bacterial load in the final disinfection (Hassen et al., 2000). Inactivation of microorganisms is proportional to the UV dose (Bitton, 2005). According to Bitton (2005), the approximate dosage for 90% inactivation of *P. aeruginosa* and *S. aureus* by UV is 5500 and 4500 μW.s/cm^2, respectively. Since the UV-dose applied in the studied WWTP is not known, it is impossible to infer about the cause of this inefficiency. Other factors such as lamp envelope, lamp ageing, water turbidity, temperature and fluid thickness can be involved in the efficiency of UV irradiation (Harris et al., 1987; Hassen et al., 2000; Qualls et al., 1985).

Importantly, the samples were collected during rainy weather, except for the sample from 17 June. According to the study, already mentioned, by Martins da Costa et al., (2006) the worst

performances of WWTP in the reduction of bacterial load occurred on rainy days, due to the increased flow rate.

With regard to results for the differentiation of *P. aeruginosa* from *P. fluorescens* and *P. putida* through of incubation at different temperatures (37 °C and 42 °C), it was observed that the majority of the populations grown in CA medium at 37 °C were *P. aeruginosa*. The difference of log between the both temperatures, with exception of sample from 28 April, is less than 1-log. The sample from 28 April at 42 °C did not result in bacterial load of *P. aeruginosa* in AD. In the same sample, the total elimination of *S. aureus* was also observed. This can indicate that disinfection was working properly in this day, since it total elimination of both pathogens under study was observed. Nevertheless, total bacterial load and load considering *Pseudomonas* other than *P. aeruginosa* remained high.

However, as mentioned previously, the presence of *P. aeruginosa* at the end of disinfection in the other samples is according to a study conducted by Hassen et al., (2000), where this bacterium was shown to be the most resistant to UV disinfection. In the case of *S. aureus*, only in the sample from 20 May its presence is verified in the final effluent. This is in line with the study by Darby et al., (1995) in which *Staphylococcus* bacteria strains are more sensitive to UV radiation than to chlorination. It is necessary to emphasize that these four samples cannot be considered representative of the efficiency of WWTP and it is, therefore, required to collect and analyse more samples.

4.2. Isolation of bacteria

In order to isolate the bacteria, the samples were filtered and the resulting membranes were placed on Petri plates containing selective solid media and biochemistry tests were used for confirmation. As mentioned previously, the methods used for the identification and isolation of presumable *P. aeruginosa*, have been adapted and optimized due to the difficulties placed by conventional methods of isolation. Table 3 describes the morphological characteristics of *P. aeruginosa* isolates and examples of some isolates are shown in Figure 8.

Table 3| Morphological features found in isolates *P. aeruginosa* (PA isolates) colonies observed on CA, at 42 °C.

		Isolate	Form	Elevation	Margin	Size	Colour	Opacity
31 March	RS*	PA isolate 1	Circular	Raised	Entire	Small	yellow	Opaque
		PA isolate 2	Circular	Raised	Entire	Small	Green	Opaque
		PA isolate 3	Circular	Raised	Entire	Small	Green	Opaque
	AD*	PA isolate 4	Circular	Flat	Entire	Small	White	Translucent
		PA isolate 5	Circular	Flat	Entire	Small	White	Translucent
28 April	AD*	PA isolate 6	Circular	Raised	Entire	Small	White	Translucent
		PA isolate 7	Circular	Flat	Entire	Medium	White	Opaque
		PA isolate 8	Circular	Flat	Entire	Small	White	Translucent
20 May	RS*	PA isolate 9	Circular	Flat	Entire	Small	White	Opaque
		PA isolate 10	Circular	Flat	Entire	Small	White	Opaque
		PA isolate 11	Circular	Flat	Entire	Small	White	Opaque
		PA isolate 12	Circular	Flat	Entire	Small	yellow-Green	Opaque
		PA isolate 13	Circular	Flat	Entire	Small	Green	Opaque
	BD*	PA isolate 14	Circular	Flat	Entire	Small	Green	Opaque
		PA isolate 15	Circular	Flat	Entire	Medium	Green	Opaque
17 June	RS*	PA isolate 16	Circular	Flat	Entire	Medium	Green	Opaque
		PA isolate 17	Circular	Flat	Entire	Large	White	Opaque
		PA isolate 18	Circular	Flat	Entire	Small	Green	Opaque
	BD*	PA isolate 19	Circular	Flat	Entire	Large	Green	Opaque
		PA isolate 20	Circular	Flat	Rhizoid	Large	Green	Opaque
		PA isolate 21	Circular	Flat	Entire	Large	Green	Opaque
	AD*	PA isolate 22	Circular	Flat	Entire	Medium	Green	Opaque
		PA isolate 23	Circular	Flat	Entire	Medium	Green	Opaque
		PA isolate 24	Circular	Flat	Entire	Small	White	Opaque

*RS – raw sewage; BD – before disinfection; and AD – after disinfection.

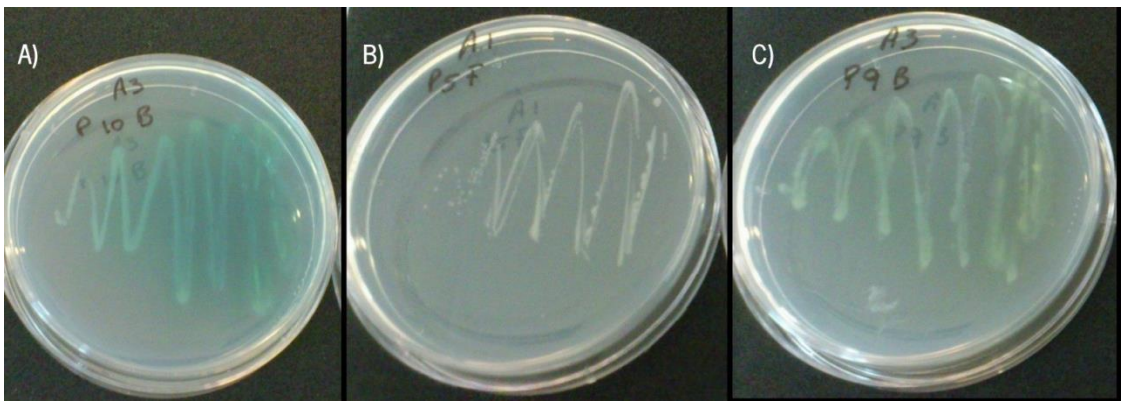

Figure 8| Isolates *P. aeruginosa* (PA isolates) colonies grown on CA plates, overnight at 37 °C. A) green colonies of PA isolate 10 ; B) white colonies of PA isolate 5; and C) yellow-green colonies of PA isolate 9.

The morphological characteristics observed in colonies of *P. aeruginosa* selected in CA medium at 42 °C are shown in Table 3. The initial choice of the colonies grown in CA medium at 42 °C

was random. In order to have three final colonies of *P. aeruginosa*, ten colonies were initially selected in each sample (SW, AD and BD). However, some of the samples had no colonies of *P. aeruginosa* since those initial selected colonies were oxidase-negative. Most colonies have identical morphological characteristics. The circular form and the entire margin are observable in all colonies (except for *P. aeruginosa* isolate 20 with a rhizoid margin).

The elevation, size and opacity varied between: flat/raised, small/medium/large and opaque/translucent, respectively. Regarding the colony colour, three different colours were observed: white, green and yellow-green (Figure 8). The colour of the colonies is in accordance with the literature. The green colour of colonies indicates that the isolate is a pyocyanin producer (considered a virulence factor). Yellow colonies are producing fluorescein, and white colonies are non-pigmented strains (Norberto, 2008; Wilson et al., 1988). As stated above, white colonies normally correspond to environmental strains of *P. aeruginosa* (Norberto, 2008).

Morphological characteristics of *S. aureus* selected strains are described in Table 4 and examples of some isolates are shown in Figure 9.

Table 4| Morphological features found in isolates *S. aureus* (SA isolates) colonies observed on MSA, at 37 °C.

		Isolate	Form	Elevation	Margin	Size	Colour	Opacity
31 March	RS*	SA isolate 1	Circular	Flat	Entire	Small	Yellow	Opaque
		SA isolate 2	Circular	Flat	Entire	Small	Yellow	Opaque
		SA isolate 3	Circular	Flat	Entire	Small	Yellow	Opaque
	BD*	SA isolate 4	Circular	Flat	Entire	Large	Yellow	Translucent
		SA isolate 5	Circular	Flat	Entire	large	Yellow	Opaque
		SA isolate 6	Circular	Flat	Entire	Small	Yellow	Opaque
	AD*	SA isolate 7	Circular	Flat	Entire	Medium	Yellow	Opaque
		SA isolate 8	Circular	Flat	Entire	Small	Yellow	Translucent
		SA isolate 9	Circular	Flat	Entire	Medium	Yellow	Translucent
28 April	RS*	SA isolate 10	Circular	Flat	Entire	Medium	Yellow	Opaque
		SA isolate 11	Circular	Flat	Entire	Medium	Yellow	Opaque
		SA isolate 12	Circular	Flat	Entire	Large	Yellow	Opaque
	BD*	SA isolate 13	Circular	Flat	Entire	Large	Yellow	Translucent
		SA isolate 14	Circular	Flat	Entire	Large	Yellow	Opaque
		SA isolate 15	Circular	Flat	Entire	Small	Yellow	Opaque
	AD*	SA isolate 16	Circular	Flat	Entire	Medium	Yellow	Opaque
		SA isolate 17	Circular	Flat	Entire	Medium	Yellow	Opaque
		SA isolate 18	Circular	Flat	Entire	Small	Yellow	Opaque

*RS – raw sewage; BD – before disinfection; and AD – after disinfection.

Table 4| Morphological features found in isolates *S. aureus* (SA isolates) colonies observed on MSA, at 37 °C (continued).

		Isolate	Form	Elevation	Margin	Size	Colour	Opacity
20 May	RS*	SA isolate 19	Circular	Flat	Entire	Small	Yellow	Opaque
		SA isolate 20	Circular	Flat	Entire	Small	Yellow	Translucent
		SA isolate 21	Circular	Flat	Entire	Small	Yellow	Opaque
	BD*	SA isolate 22	Circular	Flat	Entire	Small	Yellow	Opaque
		SA isolate 23	Circular	Flat	Entire	Small	Yellow	Opaque
		SA isolate 24	Circular	Flat	Entire	Small	Yellow	Translucent
	AD*	SA isolate 25	Circular	Raised	Entire	Medium	Yellow	Opaque
		SA isolate 26	Circular	Raised	Entire	Small	Yellow	Opaque
		SA isolate 27	Circular	Raised	Entire	Small	Yellow	Opaque
17 June	RS*	SA isolate 28	Circular	Flat	Entire	Small	Yellow	Opaque
		SA isolate 29	Circular	Flat	Entire	Small	Yellow	Opaque
		SA isolate 30	Circular	Flat	Entire	Small	Yellow	Opaque
	BD*	SA isolate 31	Circular	Flat	Entire	Small	Yellow	Opaque
		SA isolate 32	Circular	Flat	Entire	Large	Yellow	Translucent
		SA isolate 33	Circular	Flat	Entire	Medium	Yellow	Opaque
	AD*	SA isolate 34	Circular	Flat	Entire	Small	Yellow	Opaque
		SA isolate 35	Circular	Flat	Entire	Small	Yellow	Opaque
		SA isolate 36	Circular	Flat	Entire	Medium	Yellow	Opaque

*RS – raw sewage; BD – before disinfection; and AD – after disinfection.

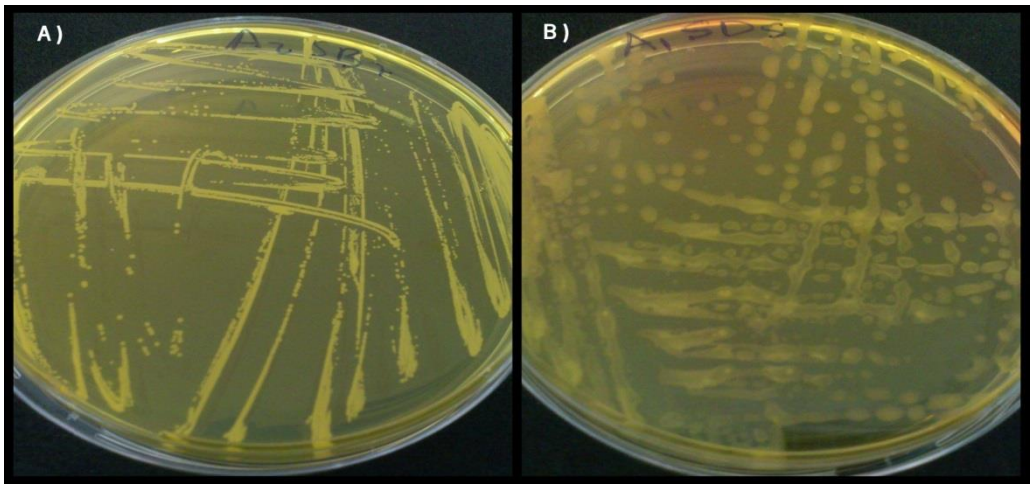

Figure 9| Isolates *S. aureus* (SA isolates) colonies grown on MSA plates, overnight at 37 °C. A) yellow and opaque colonies SA isolate 12; and B) yellow and translucent colonies SA isolate 5.

Table 4 shows that most colonies of S*. aureus* have identical morphological characteristics (MSA medium at 37 °C). The initial choice of the colonies grown in MSA medium at 37 °C was also random. In order to have three final colonies of *S. aureus* in each sample (SW, AD and BD), ten colonies were initially selected. In the case of *S. aureus* isolates it was possible to select three

colonies, which were catalase and coagulase positive, from each sample. The following features were present in all colonies: circular form; entire margin; and yellow colour. The elevation, size and opacity varied between: flat/raised, small/medium/large and opaque/translucent, respectively.

In Figure 9, two types of colonies of *S. aureus* isolates are observable. Figure 9 (A) shows a typical colony of *S. aureus*, that is, small, circular and yellow. In Figure 9 (B) it is shown an atypical colony of *S. aureus* (large, circular, yellow and with a gelatinous consistency). In both plates there is mannitol fermentation (medium colour change from red to yellow). The colony shown in Figure 9 (B) has a morphology that has never been ascribed to *S. aureus* in the literature. However, as all the isolates showing this morphology are also catalase and coagulase-positive, they were considered to be *S. aureus*. In order to make a more reliable identification, it would be necessary to resort to molecular tests. The production of golden yellow pigment and mannitol fermentation of MSA medium present in all isolates is in accordance with the literature (Engelkirk and Duben-Engelkirk, 2008).

4.3. Antibiotic susceptibility testing

The resistance of bacteria to different antibiotics was tested by modified Kirby-Bauer disk diffusion method. Tables 7 and 8 (Appendix A) show the ATS results alongside with the size of inhibition zones formed in the presence of *P. aeruginosa* and *S. aureus*, respectively. In Appendix B, some photographs of isolates in MHA medium and their respective responses to antibiotics are shown (Figures 14 and 15). The ATS results are described in Tables 5 and 6 to *P. aeruginosa* and *S. aureus*, respectively.

Table 5| Antimicrobial susceptibility tests (according to EUCAST) results of *P. aeruginosa* isolates (PA isolates) for the following antibiotics: 30 µg piperacillin (PIP); 5 µg ciprofloxacin (CIP); 30 µg amikacin (AMK); 10 µg gentamicin (GENT) and 10 µg tobramycin (TOB).

		PIP	CIP	AMK	GENT	TOB
		S,R,I*	S,R,I*	S,R,I*	S,R,I*	S,R,I*
31 March						
RS**	PA isolate 1	S	S	R	S	S
	PA isolate 2	S	S	S	S	S
	PA isolate 3	S	S	R	S	S
AD**	PA isolate 4	S	I	S	S	S
	PA isolate 5	S	I	S	S	S

* S – susceptible; I – intermediate; and R – resistant.

** RS – raw sewage; BD – before disinfection; and AD – after disinfection.

Table 5| Antimicrobial susceptibility tests (according to EUCAST) results of *P. aeruginosa* isolates (PA isolates) for the following antibiotics: 30 µg piperacillin (PIP); 5 µg ciprofloxacin (CIP); 30 µg amikacin (AMK); 10 µg gentamicin (GENT) and 10 µg tobramycin (TOB) (continued).

		PIP	CIP	AMK	GENT	TOB
		S,R,I*	S,R,I*	S,R,I*	S,R,I*	S,R,I*
28 April						
AD**	PA isolate 6	S	S	S	S	S
	PA isolate 7	S	R	S	S	S
	PA isolate 8	S	S	S	S	S
20 May						
RS**	PA isolate 9	R	R	S	S	S
	PA isolate 10	S	I	S	S	S
	PA isolate 11	S	R	S	S	S
	PA isolate 12	S	R	S	S	S
	PA isolate 13	S	R	S	S	S
BD**	PA isolate 14	S	R	S	S	S
	PA isolate 15	S	R	S	S	S
17 June						
RS**	PA isolate 16	S	S	S	S	S
	PA isolate 17	S	S	S	S	S
	PA isolate 18	S	S	S	S	S
BD**	PA isolate 19	S	S	S	S	S
	PA isolate 20	S	S	S	S	S
	PA isolate 21	S	S	S	S	S
AD**	PA isolate 22	S	S	S	S	S
	PA isolate 23	S	S	S	S	S
	PA isolate 24	S	R	S	S	S

* S – susceptible; I – intermediate; and R – resistant.

** RS – raw sewage; BD – before disinfection; and AD – after disinfection.

Inhibition zone diameters were used to evaluate the susceptibility of *P. aeruginosa* isolates to antibiotics. Table 5 shows that the majority of the isolates were susceptible to the tested antibiotics. All isolates were susceptible to tobramycin and gentamicin. All isolates were shown to be susceptible to piperacillin, except for *P. aeruginosa* isolate 9. The isolates 1 and 3 were the only to show resistance to amikacin. Isolates 7, 9, 11, 12, 13, 14, 15 and 24 were shown to be resistant to ciprofloxacin, while *P. aeruginosa* isolates 4, 5 and 10 were classified as intermediate. The remaining isolates were susceptible to ciprofloxacin. Summarizing, the isolates of *P. aeruginosa* were susceptible to tobramycin and gentamycin, in general susceptible to piperacillin and amikacin and fairly resistant to ciproflaxin. Of the eleven resistances observed in *P. aeruginosa*

isolates, seven isolates belong to the RS samples (isolates 1, 3, 9, 11, 12 and 13), two to the BD samples (isolates 14 and 15) and other two to the AD samples (isolates 7 and 24).

Table 6| Antimicrobial susceptibility tests (according to EUCAST) results of *S. aureus* isolates (SA isolates) for the following antibiotics: 5 µg rifampicin (RIF); 30 µg amikacin (AMK); 30 µg chloramphenicol (CHF); 5 µg ciprofloxacin (CIP); 30 µg tetracycline (TETRA); 10 µg tobramycin (TOB); and 10 µg gentamicin (GENT).

		RIF	AMK	CHF	CIP	TETRA	TOB	GENT
		S,R,I*	S,R,I*	S,R,I*	S,R,I*	S,R,I*	S,R,I*	S,R,I*
	31 March							
RS**	SA isolate 1	S	S	S	S	S	S	S
	SA isolate 2	S	S	S	S	S	S	S
	SA isolate 3	S	S	S	R	S	S	S
BD**	SA isolate 4	R	S	R	S	S	S	S
	SA isolate 5	R	S	S	S	S	S	S
	SA isolate 6	S	S	S	S	S	S	S
AD**	SA isolate 7	R	S	S	S	S	S	S
	SA isolate 8	R	S	S	S	S	S	S
	SA isolate 9	R	S	S	S	S	S	S
28 April								
RS**	SA isolate 10	S	S	S	S	S	S	S
	SA isolate 11	S	S	S	S	S	S	S
	SA isolate 12	S	S	S	S	S	S	S
BD**	SA isolate 13	S	S	S	S	S	S	S
	SA isolate 14	S	S	S	S	S	S	S
	SA isolate 15	R	S	S	S	S	S	S
AD**	SA isolate 16	S	S	S	S	S	S	S
	SA isolate 17	S	S	S	S	S	S	S
	SA isolate 18	S	S	S	S	S	S	S
20 May								
RS**	SA isolate 19	S	S	S	S	S	S	S
	SA isolate 20	I	S	S	S	S	S	S
	SA isolate 21	S	S	S	S	S	S	S
BD**	SA isolate 22	S	S	S	S	S	S	S
	SA isolate 23	S	S	S	S	S	S	S
	SA isolate 24	S	S	S	S	S	S	S
AD**	SA isolate 25	R	S	S	S	S	S	S
	SA isolate 26	S	S	S	S	S	S	S
	SA isolate 27	R	S	S	S	S	S	S

* S – susceptible; I – intermediate; and R – resistant.

** RS – raw sewage; BD – before disinfection; and AD – after disinfection

Table 6| Antimicrobial susceptibility tests (according to EUCAST) results of *S. aureus* isolates (SA isolates) for the following antibiotics: 5 µg rifampicin (RIF); 30 µg amikacin (AMK); 30 µg chloramphenicol (CHF); 5 µg ciprofloxacin (CIP); 30 µg tetracycline (TETRA); 10 µg tobramycin (TOB); and 10 µg gentamicin (GENT) (continued).

		RIF	AMK	CHF	CIP	TETRA	TOB	GENT
		S,R,I*	S,R,I*	S,R,I*	S,R,I*	S,R,I*	S,R,I*	S,R,I*
17 June								
RS**	SA isolate 28	S	S	S	S	S	S	S
	SA isolate 29	S	S	S	S	S	S	S
	SA isolate 30	S	S	S	S	S	S	S
BD**	SA isolate 31	R	S	S	S	S	S	S
	SA isolate 32	R	S	S	S	S	S	S
	SA isolate 33	R	S	S	S	S	S	S
AD**	SA isolate 34	I	S	S	S	S	S	S
	SA isolate 35	R	S	S	S	S	S	S
	SA isolate 36	R	S	S	S	S	S	S

* S – susceptible; I – intermediate; and R – resistant.

** RS – raw sewage; BD – before disinfection; and AD – after disinfection

As happened with *P. aeruginosa* isolates, the majority of isolates of *S. aureus* were susceptible to the tested antibiotics (Table 6). All isolates were susceptible to the following antibiotics: tobramycin, gentamicin, amikacin and tetracycline. Isolates 3 and 4 were the only that showed resistance to ciprofloxacin and chloramphenicol, respectively. The isolates 4, 5, 7, 8, 9, 15, 24, 27, 32, 33, 35 and 36 were shown to be resistant to rifampicin, while *S. aureus* isolates 20 and 34 were classified as intermediate. The remaining isolates were susceptible to rifampicin. Summarizing, the *S. aureus* isolates were susceptible to tobramycin, gentamicin, amikacin and tetracycline, in general susceptible to ciprofloxacin and chloramphenicol and moderately resistant to rifampicin. Of the fifteen resistances observed in *S. aureus* isolates, only one isolate belong to the RS samples (isolate 3), seven to the BD samples (isolates 4, 5, 15, 31, 32 and 33) and other seven to the AD samples (isolates 7, 8, 9, 25, 27, 35 and 36).

Overall, both isolates of *P. aeruginosa* and *S. aureus* isolates were susceptible to most antibiotics. There was a high number of isolates in both species showing resistance to two antibiotics. Eight of the isolates *of P. aeruginosa* presented resistance to ciprofloxacin and three of them were considered intermediate. For rifampicin, thirteen isolates of *S. aureus* presented resistance whereas two of them were considered intermediate. However, according to the literature, these species are normally susceptible to these antibiotics. In the case of *P. aeruginosa*, a study conducted by Ndip et al., (2005) showed that 98% of clinical isolates were susceptible to

ciprofloxacin. Gales et al., (2001) has documented ciprofloxacin as the most potent agent available in oral form for the treatment of *P. aeruginosa* infections. *S. aureus* is susceptible to rifampicin, but it is able to acquire resistance through mutations (Liu et al., 2005; Schmitz et al., 2000). According to Traczewski et al., (1983) rifampicin is used in combination with other antibiotics as a last resource to treat serious infections. However, rifampicin cannot be used alone because bacteria easily develop resistance.

Thus, two possible explanations for the high number of isolates resistant to ciprofloxacin and rifampicin are: transfer of resistant genes or the presence of these antibiotics in WWTP in sub-inhibitory concentrations. As already mentioned, the WWTP consist in a concoction of bacteria, nutrients, and antimicrobial agents in sub-inhibitory concentrations, which can promote the proliferation of resistant bacteria and transfer of resistant genes to susceptible bacteria (Kruse and Sørum, 1994; Lindberg et al., 2004; Mach and Grimes, 1982; Poté et al., 2003). It is more probable that this occurred in the case of *S. aureus* and not in the case of *P. aeruginosa* as in this case more resistances were found in the sewage than in the final effluents, before and after disinfection. Anyway, these conclusions can be affected by the choice of the colonies to be isolated, a step that was as randomly as possible but could have left behind other colonies that would lead to different perspectives.

Antibiotics can be classified according to their spectrum of action: narrow-spectrum antibiotics, which are effective against one or very few types of microorganisms, and broad-spectrum antibiotics, which are effective against a wide range of microorganisms (Lyons, 2011). Both rifampicin and ciprofloxacin are considered to be broad-spectrum antibiotics (Fass, 1990; Llorens-Terol and Busquets, 1980), and therefore their presence in WWTP is even more concerning. The possible presence of pathogenic bacteria sensitive to these antibiotics in wastewater, as well as the presence of these antibiotics in sub-inhibitory concentration, may result in the bacteria acquiring resistance (Jury et al., 2011). According to the study realized by Zhang and Li (2013), the presence of 56 types of antibiotics belonging to six different classes has been detected in WWTP in the world, with concentrations ranging between μg/L to ng/L. Because of that, the presence of rifampicin and ciprofloxacin in these wastewaters is likely to occur. Another possibility is that non-pathogenic bacteria acquire resistance to these antibiotics and transfer the resistance genes to pathogenic bacteria via horizontal gene transfer (Jury et al., 2011).

The location of the resistant isolates in the samples was taken into account. Analyzing in the overall, the results obtained showed a significant presence of resistant isolates, in both bacteria, in

the samples corresponding to AD. It is also seen that despite the choice of colonies to be random, the number of resistant isolates in BD samples is equal AD. It can be conclude that besides disinfection do not show efficacy in reducing the bacterial load, allows the survival and release of pathogenic bacteria to the environment. Thus, it is very important to invest on the optimization of WWTP and on new treatments able to effectively eliminate ARB and antibiotics. It was also observed that in *S. aureus* isolates, there was an increase of resistance between the RS samples for BD and AD samples This increase can be justified with the transfer of resistant genes or the presence of these antibiotics in WWTP in sub-inhibitory concentrations, as mentioned above, but to be certain extensive isolations should have been made.

Only the *P. aeruginosa* isolate 9 presented resistance to two antibiotics of different classes. Isolate *S. aureus* 4 presented resistance to two antibiotics, chloramphenicol and rifampicin, but they belong to the same class. Therefore, they were not classified as multidrug-resistant (MDR) since resistance to three or more antimicrobial classes was not recorded. This is important since they are potentially pathogenic and resistant bacteria. It should be emphasized that some of the most important antibiotics such as vancomycin and methicillin, for *S. aureus*, and a carbapenem, for *P. aeruginosa*, were not tested. It would be important to test these antibiotics to identify possible methicillin-resistant *S. aureus*, vancomycin-resistant *S. aureus*, as well as carbapenem-resistant *P. aeruginosa.* These microorganisms are considered with high morbidity and mortality rates due to multiple mutations endowing high levels of resistance to antibiotic classes specifically recommended for their treatment (Davies and Davies, 2010).

4.4. Biofilm formation

The ability to form biofilm is a sign of pathogenicity of a bacterium. Thus, the ability of each isolated bacteria to form biofilm was assessed by determining the amount of biofilm biomass accumulated (through CV method) and the number of the biofilm-associated cells. The amount of biofilm biomass of all *P. aeruginosa* isolates is illustrated in Figure 10.

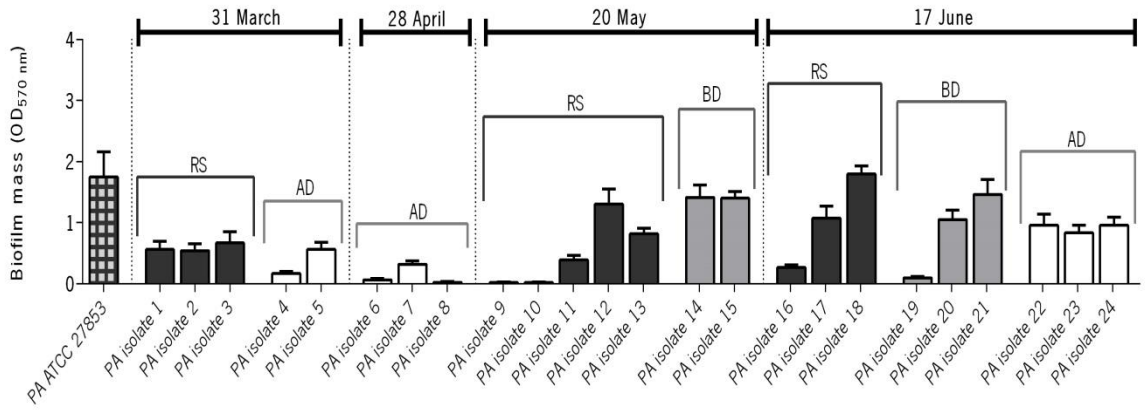

Figure 10| Biofilm mass ($OD_{570\ nm}$) formed by *P. aeruginosa* ATCC 27853 (PA ATCC 27853) and *P. aeruginosa* isolates (PA isolates) of four samples, from three different locations: raw sewage (RS); before disinfection (BD); after disinfection (AD). Growth during 24 h at 37 °C with an agitation of 120 rpm. Bars are representative of the average biofilm biomass from three independent assays.

All isolates were compared to control *P. aeruginosa* ATCC 27853. The isolates showed absorbance in the range of 0.02 to 1.8. The isolate with the highest ability to form biofilm was the isolate 18, followed by the isolates 21, 14, 15 and 12. Isolates with less biofilm formation capacity were the isolates 6, 8, 9, 10 and 19 ($p > 0.0001$).

The viable and cultivable biofilm-cells of all *P. aeruginosa* isolates are illustrated in Figure 11.

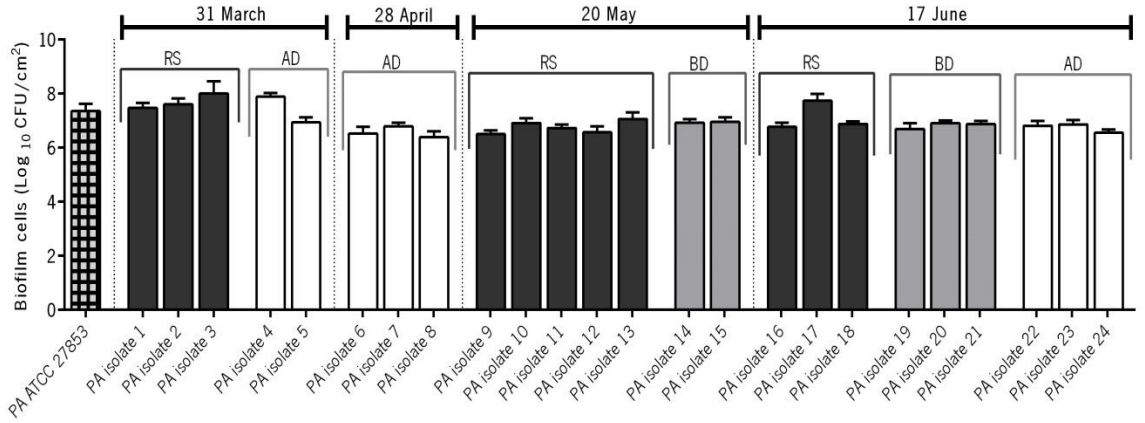

Figure 11| Biofilm cell (Log_{10} CFU/cm²) quantification of *P. aeruginosa* ATCC 27853 (PA ATCC 27853) and *P. aeruginosa* isolates (PA isolates) of four samples, from three different locations: raw sewage (RS); before disinfection (BD); after disinfection (AD). Growth during 24 h at 37 °C with an agitation of 120 rpm. Bars are representative of the average biofilm biomass from three independent assays.

Figure 11 point out that the biofilms formed by all the isolates had viable and cultivable cells between 8 and 6 log units. The isolates that had a higher cell counts were the *P. aeruginosa* isolates 1, 2, 3, 4 and 17, while the *P. aeruginosa* isolates 6, 8, 9, 12 and 24 ($p > 0.0001$) were the ones with a lower cell number. As CV is a non-specific dye that binds to negatively charged

molecules found in the extracellular matrix and in both living and dead cells (Extremina et al., 2011), another method such as CFU quantification must be used in order to assess cell viability. The number of colonies counted by the CFU method provides information about the number of viable and cultivable cells present in the biofilm, and it is thus more specific than the use of the CV method. The number of viable and cultivable cells is not, however, directly related with biofilm-formation ability. For example, isolate 12 seems to be a good biofilm producer, when compared with other isolates, however its low viable cell number may be indicative of a biofilm with a higher EPS content.

The isolates 8, 9 and 6 appear to be no biofilm formers because they revealed to show almost no $OD_{570\ nm}$ (0.02). However, they have a number of viable cells of 6 log units. This may be due to the detection limit of the colorimetric method.

The ability to form biofilm was also determined to all *S. aureus* isolates. The amount of biomass formed and biofilm cell quantification results are illustrated in Figures 12 and 13, respectively.

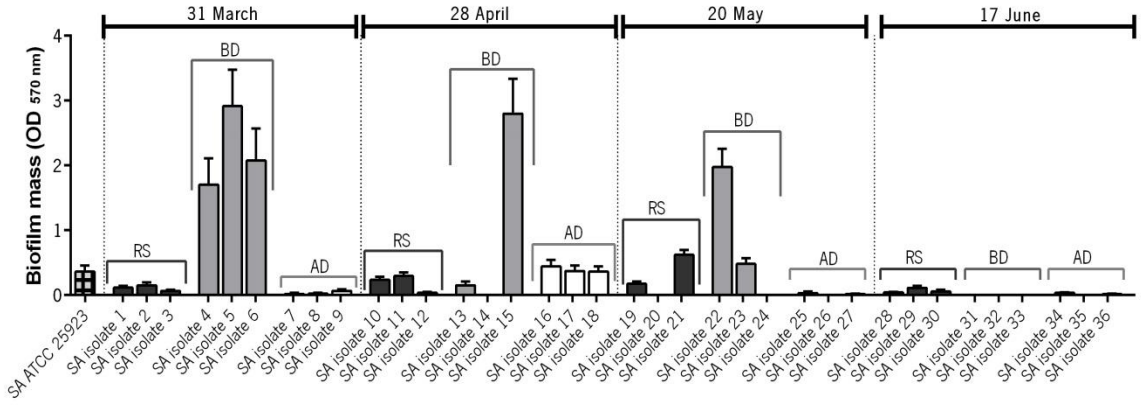

Figure 12| Biofilm mass ($OD_{570\ nm}$) formed by *S. aureus* ATCC 25923 (SA ATCC 25923) and *S. aureus* isolates (SA isolates) of four samples from three different locations: raw sewage (RS); before disinfection (BD); after disinfection (AD). Growth during 24 h at 37 °C with an agitation of 120 rpm. Bars are representative of the average biofilm biomass from three independent assays.

All isolates were compared to control *S. aureus* ATCC 25923. The isolates showed absorbances in the range of 0 to 2.92. The isolates with a larger biofilm formation ability were isolates 5 ($p > 0.01$), followed by the isolates: 15 ($p > 0.01$), 6, 22 and 4 ($OD_{570\ nm} = 1.70$). The isolates with less biofilm formation capacity ($OD_{570\ nm} \leq 0.02$) were isolates 7, 8, 27 and 36 ($p > 0.0001$). Eight *S. aureus* isolates (14, 20, 24, 26, 31, 32, 33 and 35) can be considered as no biofilm producers as the values of absorbance are close to 0 ($p > 0.0001$)

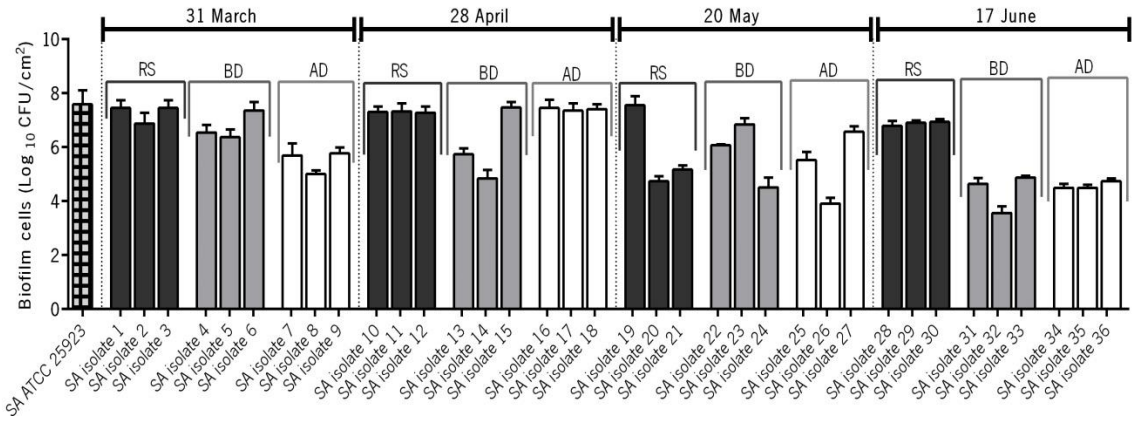

Figure 13 | Biofilm cell (Log_{10} CFU/cm²) quantification of *S. aureus* ATCC 25923 (SA ATCC 25923) and *S. aureus* isolates (SA isolates) of four samples from three different locations: raw sewage (RS); before disinfection (BD); after disinfection (AD). Growth during 24 h at 37 °C with an agitation of 120 rpm. Bars are representative of the average biofilm biomass from three independent assays.

Figure 13 represents the number of viable and cultivable cells present in the biofilms formed by *S. aureus* isolates. These biofilms have viable cell numbers between 7.5 and 3.5 log units. The five isolates with a higher cell count were isolates 1, 3, 16, 15 and 19 (7.55 – 7.46 log units), while the *S. aureus* isolates 24, 26, 32, 34 and 35 ($p > 0.0001$) were the ones with a lower cell number (3.56 – 4.51 log units). The isolate 5 had the highest biofilm-forming ability, but it was not the isolate which had the highest number of viable cells (6.34 log units), indicating that it can be a strong producer of EPS matrix. The same trend can be speculated for isolates 22 and 4. The isolates 15 and 16 had also a high ability to form biofilm withhigh viable cell number (7.74 and 7.36 –log units). Isolates 19, 1 and 3, despite the high number of viable cells (7.55 - 7.46), they presented low biofilm formation capacity, with $OD_{570\ nm}$ values between 0.18 and 0.06. This appears to indicate that these isolates are weak matrix producers.

The isolates that have absorbance close to zero also had smaller numbers of viable cells (3.56 – 4.88 log units). The fact that absorbance is near to zero does not mean that there is no microbial adhesion. A possible explanation for this result may be related to the size of *S. aureus* cell. According to Sulaeman et al. (2010) and Joshua et al. (2006), the CV method could not be applied to Campylobacter due to the size of its cells and to the limitations of this method. For example, Tresse et al. (2006) refers that to *Listeria monocytogenes*, this technique requires at least 10^7 attached cells per well in a 96-well microtitre plate to obtain a detection signal. Thus, considering that *S. aureus* cells are small and the viable cell numbers are less than 7 log units, it can be speculated that there is bacterial adhesion that is not detected due to the low resolution of the

method. It should be noticed that CV staining requires washing steps which could remove parts of the attached biofilm (Gunther IV and Chen, 2009).

By comparing biofilms formed by *P. aeruginosa* and *S. aureus* isolates, it is possible to verify that, in general, the isolates that had both higher biofilm formation capacity and larger number of viable and cultivable cells were those formed by *P. aeruginosa*. In both species it was not observed any relationship between biofilm formation and the three different locations in the WWTP where the samples were collected. Isolates from AD were expected to show greater biofilm formation, when compared with BD and SW, since these isolates were exposed to additional stressful environmental conditions, in this case the ultraviolet radiation that might have led to an increase in the level of biofilm formation. This is supported by studies already carried out (Mah and O'Toole 2001; Reuter et al. 2010; Seufferheld et al. 2008). However, the *in vitro* biofilm-forming capacity does not necessarily correlate to *in vivo* biofilm production (Bendouah et al., 2006).

Biofilm formation is an important virulence factor and it is responsible for a variety of infections. The bacteria entrapped in the EPS matrix are more difficult to remove compared with planktonic form. Biofilms have the ability to resist host defences and are resistant to many antibiotics. *P. aeruginosa* and *S. aureus* are common nosocomial pathogens responsible for biofilm-associated infections and so it is important to study the ability of these bacteria to form biofilms.

Apparently, no relation seems to exist between a high capacity of biofilm formation and a high level of resistance to antibiotics. In effect, the values from biofilm formation of *S. aureus* and *P. aeruginosa* isolates that showed resistance vary between a $OD_{570\ nm}$ range from 3 to 0. In the case of *P. aeruginosa* isolates, 9 out of 10 which showed resistance presented an absorbance above 0.2. Regarding *S. aureus* isolates, only 3 of 14 which showed resistance obtained an absorbance value above 0.2. The *P. aeruginosa* isolate 9, which was resistant to two classes of antibiotics, showed to be the weakest producer of biofilm, displaying an absorbance value of 0.02. Antibiograms of biofilm-associated cells would be a possible method to confirm the possible relation between these two factors.

CHAPTER V| CONCLUSIONS AND FUTURE PRESPECTIVES

5.1. Conclusions

Water is essential to life on earth. It is retrieved from the environment in order to fulfill several human needs, such domestic, industrial, and hospital activities, being returned to the environment afterwards. Nevertheless, before the water can be safely returned, it is required to be properly treated. Pollutants must be removed from the water and this process occurs in the WWTP. Therefore, WWTP have a crucial role regarding public health and the preservation of water resources. However, several recent studies show that the current WWTP facilities are not well prepared to effectively eliminate antibiotics and bacteria, and may even provide a favorable environment for the development of antibiotic-resistant bacteria and their dissemination to the surrounding environment.

The current work was focused on the study of the efficacy of one WWTP, with a tertiary treatment disinfection system, in the reduction of the bacterial load present at the final effluent, and particularly the reduction of the bacterial loads of *S. aureus* and *P. aeruginosa*. Additionally, several isolates of *S. aureus* and *P. aeruginosa* were collected to further inspect their susceptibility to different selected antibiotics and the ability to form biofilm. In short, with this work the following conclusions were drawn:

- In general, the UV disinfection did not display effective results regarding the reduction of bacterial load, with the exception of one sample (31 March). In general, the bacterial load of *P. aeruginosa* remained high and almost unchanged after disinfection. In regard to the bacterial load of *S. aureus*, it exhibited significant reduction, and its presence was only verified at the final effluent in one sample (20 May). Therefore, *P. aeruginosa* seems to be more resistant to disinfection with UV radiation than *S. aureus*, an observation corroborating previous work.
- Regarding antibiotic susceptibility tests, the majority of the isolates displayed responsiveness, except for two of the tested antibiotics. Eight isolates of *P. aeruginosa* were resistant to ciprofloxacin, and thirteen of the isolates of *S. aureus* were resistant to rifampicin. This can be explained either by a potential transference of resistance genes or by the presence of these antibiotics in sub-inhibitory concentrations at the WWTP, but it seems more probably to be the case of the resistance of *S. aureus* but not of *P. aeruginosa*. The location of the resistant isolates in the samples is concern, once the results obtained showed a significant presence of resistant isolates in BD and AD samples.

It means that the WWTP are not effective in the elimination of antibiotics and resistant genes as like allows the survival and release of ARB to the environment, besides the low efficiency on reduction of registered bacterial load. Only one isolate (a *P.aeruginosa* isolate) appeared to be resistant to two classes of antibiotics (piperacicllin and ciprofloxacin).

- The amount of biofilm formation, both from *P. aeruginosa* and *S. aureus* isolates, varied significantly. Due to this variation, it was not possible to establish a relationship between the amount of biofilm formation and the site at WWTP where the isolated colony was collected. It was possible to verify that despite the fact that the number of isolates was not the same, the isolates of *P. aeruginosa* had a greater capacity of biofilm formation when compared with *S. aureus*. Apparently, no relation seems to exist between a high level of resistance to antibiotics and a high capacity of biofilm formation.

5.2. Future Perspectives

According to the results obtained, it would be interesting for a future work to:

- Collect more samples in order to obtain a representative sample and thus to evaluate more accurately the disinfection capacity of this WWTP;
- Compare the antibiotic susceptibility profiles of the isolates in planktonic form to the profiles of bacteria in biofilm form;
- Test other antibiotics in order to obtain a more complete susceptibility profile.

For a future research work it would be interesting to quantify the presence of other pathogenic microorganisms, the presence of antibiotics and genes for antibiotic resistance, as well as to evaluate the efficiency of other disinfection methods already applied at the WWTP, with the main goal of verifying which method is more effective at reducing these three components.

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APPENDICES

Appendix A

Tables 7 and 8 show the antimicrobial susceptibility test results alongside with the size of inhibition zones formed in the presence of *P. aeruginosa* and *S. aureus*, respectively.

Table 7 | Antimicrobial susceptibility tests (according to EUCAST) results of *P. aeruginosa* isolates for the following antibiotics: 30 µg piperacillin (PIP); 5 µg ciprofloxacin (CIP); 30 µg amikacin (AMK); 10 µg gentamicin (GENT) and 10 µg tobramycin (TOB).

		PIP		CIP		AMK		GENT		TOB	
		Diameter	S,R,I*	Diameter	S,R,I*	Diameter	S,R,I*	Diameter	S,R,I*	Diameter	S,R,I*
31 March											
RS**	PA isolate 1	2,7	S	3,2	S	0,6	R	1,9	S	2,4	S
	PA isolate 2	2,4	S	3,9	S	2,8	S	2,5	S	2,4	S
	PA isolate 3	2,4	S	3,7	S	0,6	R	2,6	S	2,4	S
AD**	PA isolate 4	2,2	S	2,2	I	2,4	S	2,3	S	2,1	S
	PA isolate 5	2,2	S	2,4	I	2,4	S	2,1	S	2,1	S
28 April											
AD**	PA isolate 6	2,2	S	3,4	S	3,1	S	2,5	S	2,3	S
	PA isolate 7	2,2	S	2,1	R	2,5	S	2,4	S	2,8	S
	PA isolate 8	2,3	S	3,3	S	2,8	S	2,7	S	2,3	S
20 May											
RS**	PA isolate 9	1,3	R	1,0	R	2,3	S	2,1	S	2,0	S
	PA isolate 10	2,5	S	2,2	I	2,1	S	2,0	S	1,9	S
	PA isolate 11	2,1	S	0,9	R	2,5	S	2,3	S	2,3	S
	PA isolate 12	2,4	S	3,2	R	2,9	S	2,6	S	2,5	S
	PA isolate 13	2,3	S	3,0	R	2,9	S	2,4	S	2,4	S
BD**	PA isolate 14	2,2	S	3,2	R	2,8	S	2,5	S	2,4	S
	PA isolate 15	2,3	S	3,3	R	2,8	S	2,5	S	2,3	S
17 June											
RS**	PA isolate 16	2,9	S	2,6	S	2,5	S	2,3	S	2,4	S
	PA isolate 17	2,6	S	3,3	S	3,0	S	2,6	S	2,6	S
	PA isolate 18	2,5	S	3,1	S	3,0	S	2,6	S	2,5	S
BD**	PA isolate 19	2,3	S	3,4	S	2,7	S	2,4	S	2,4	S
	PA isolate 20	2,4	S	3,5	S	3,0	S	2,4	S	2,4	S
	PA isolate 21	2,6	S	3,3	S	2,8	S	2,4	S	2,4	S
AD**	PA isolate 22	2,5	S	3,1	S	2,8	S	2,6	S	2,5	S
	PA isolate 23	2,8	S	3,3	S	2,9	S	2,7	S	2,5	S
	PA isolate 24	2,6	S	0,6	R	3,1	S	2,7	S	2,7	S

* S – susceptible; I – intermediate; and R – resistant.

** RS – raw sewage; BD – before disinfection; and AD – after disinfection

Table 8| Antimicrobial susceptibility tests (according to EUCAST) results of *S. aureus* isolates (SA isolates) for the following antibiotics: 5 µg rifampicin (RIF); 30 µg amikacin (AMK); 30 µg chloramphenicol (CHF); 5 µg ciprofloxacin (CIP); 30 µg tetracycline (TETRA); 10 µg tobramycin (TOB); and 10 µg gentamicin (GENT).

		RIF		AMK		CHF		CIP		TETRA		TOB		GENT	
		diameter	S,R,I*	diameter	S,R,I*	diameter	S,R,I*	diameter	S,R,I*	diameter	S,R,I*	diameter	S,R,I*	diameter	S,R,I*
	31 March														
RS**	SA isolate 1	3,1	S	2,1	S	2,2	S	2,8	S	2,9	S	2,1	S	2,2	S
	SA isolate 2	3,0	S	2,0	S	2,1	S	2,5	S	2,8	S	2,2	S	2,1	S
	SA isolate 3	3,3	S	2,2	S	2,4	S	1,0	R	2,8	S	2,1	S	2,0	S
BD**	SA isolate 4	1,7	R	2,4	S	1,5	R	2,9	S	2,2	S	2,6	S	2,6	S
	SA isolate 5	2,2	R	2,9	S	2,0	S	3,2	S	2,2	S	2,9	S	2,8	S
	SA isolate 6	3,7	S	3,0	S	2,6	S	2,7	S	3,5	S	3,4	S	3,0	S
AD**	SA isolate 7	2,0	R	2,7	S	2,2	S	2,8	S	3,0	S	2,6	S	2,5	S
	SA isolate 8	2,3	R	2,8	S	2,6	S	2,7	S	3,2	S	2,6	S	2,7	S
	SA isolate 9	2,2	R	2,8	S	2,2	S	3,0	S	3,4	S	2,6	S	2,6	S
	28 April														
RS**	SA isolate 10	2,9	S	2,0	S	2,2	S	2,6	S	2,7	S	2,0	S	1,9	S
	SA isolate 11	3,0	S	2,2	S	2,4	S	2,7	S	3,8	S	3,2	S	2,9	S
	SA isolate 12	3,6	S	3,1	S	2,2	S	2,4	S	2,6	S	2,0	S	2,9	S
BD**	SA isolate 13	3,5	S	2,8	S	2,6	S	2,7	S	3,4	S	2,7	S	2,4	S
	SA isolate 14	3,0	S	2,8	S	2,9	S	3,6	S	3,7	S	2,7	S	2,7	S
	SA isolate 15	2,3	R	2,8	S	2,3	S	3,2	S	3,0	S	2,5	S	2,2	S
AD**	SA isolate 16	3,1	S	2,4	S	2,2	S	2,7	S	2,8	S	2,3	S	2,2	S
	SA isolate 17	4,2	S	3,1	S	2,8	S	2,8	S	3,5	S	3,0	S	2,2	S
	SA isolate 18	3,3	S	2,3	S	2,5	S	2,7	S	3,3	S	3,1	S	2,3	S

* S – susceptible; I – intermediate; and R – resistant.

** RS – raw sewage; BD – before disinfection; and AD – after disinfection

Table 8 | Antimicrobial susceptibility tests (according to EUCAST) results of *S. aureus* isolates (SA isolates) for the following antibiotics: 5 µg rifampicin (RIF); 30 µg amikacin (AMK); 30 µg chloramphenicol (CHF); 5 µg ciprofloxacin (CIP); 30 µg tetracycline (TETRA); 10 µg tobramycin (TOB); and 10 µg gentamicin (GENT) (continued).

		RIF		AMK		CHF		CIP		TETRA		TOB		GENT	
		diameter	S,R,I*	diameter	S,R,I*	diameter	S,R,I*	diameter	S,R,I*	diameter	S,R,I*	diameter	S,R,I*	diameter	S,R,I*
20 May															
RS**	SA isolate 19	3,1	S	2,1	S	2,4	S	2,5	S	2,8	S	2,1	S	2	S
	SA isolate 20	2,4	I	2,3	S	2,7	S	3,3	S	3,4	S	2,2	S	2,2	S
	SA isolate 21	3,2	S	2,8	S	2,6	S	2,8	S	2,9	S	2,8	S	2,7	S
BD**	SA isolate 22	3,2	S	2,6	S	2,5	S	2,2	S	3,0	S	2,6	S	2,3	S
	SA isolate 23	3,1	S	2,1	S	2,4	S	2,3	S	2,8	S	2,1	S	2,0	S
	SA isolate 24	3,0	S	2,3	S	2,9	S	3,2	S	2,3	S	3,5	S	2,2	S
AD**	SA isolate 25	2,0	R	2,5	S	1,9	S	2,5	S	2,3	S	2,4	S	2,3	S
	SA isolate 26	2,9	S	2,4	S	1,8	S	3,2	S	3,4	S	3,4	S	2,3	S
	SA isolate 27	2,2	R	2,8	S	1,9	S	3,2	S	3,0	S	2,6	S	2,5	S
17 June															
RS**	SA isolate 28	3,2	S	2,0	S	2,4	S	2,7	S	3,0	S	2,2	S	2,0	S
	SA isolate 29	3,1	S	2,1	S	2,5	S	2,9	S	2,9	S	2,2	S	2,1	S
	SA isolate 30	3,3	S	2,0	S	2,2	S	2,5	S	2,9	S	2,2	S	2,0	S
BD**	SA isolate 31	2,1	R	2,5	S	2,0	S	3,2	S	3,0	S	2,3	S	2,3	S
	SA isolate 32	2,3	R	2,8	S	1,9	S	3,3	S	3,8	S	2,9	S	2,8	S
	SA isolate 33	2,1	R	2,7	S	2,4	S	3,3	S	3,3	S	2,7	S	2,6	S
AD**	SA isolate 34	2,4	I	2,8	S	2,2	S	3,2	S	3,3	S	2,7	S	2,6	S
	SA isolate 35	2,2	R	2,9	S	2,1	S	3,3	S	3,3	S	2,8	S	2,7	S
	SA isolate 36	2,2	R	2,8	S	2,2	S	2,9	S	3,4	S	2,8	S	2,7	S

* S – susceptible; I – intermediate; and R – resistant.

** RS – raw sewage; BD – before disinfection; and AD – after disinfection

Appendix B

Some photographs of isolates in MHA medium and their respective responses to antibiotics are shown in Figures 14 and15.

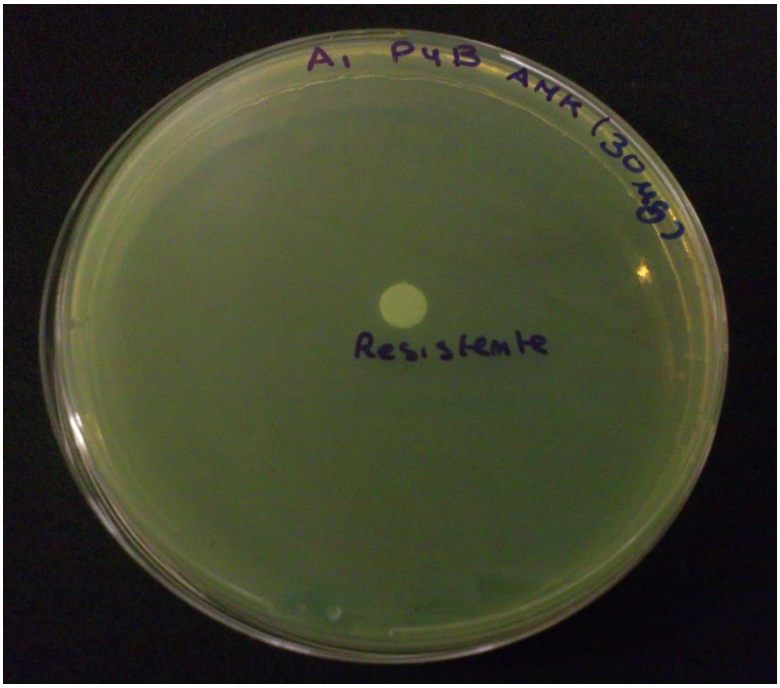

Figure 14| Antibiotic susceptibility profiles of *P. aeruginosa* isolate 3 to amikacin (30 µg). Growth in MHA medium for 18h, at 37 °C.

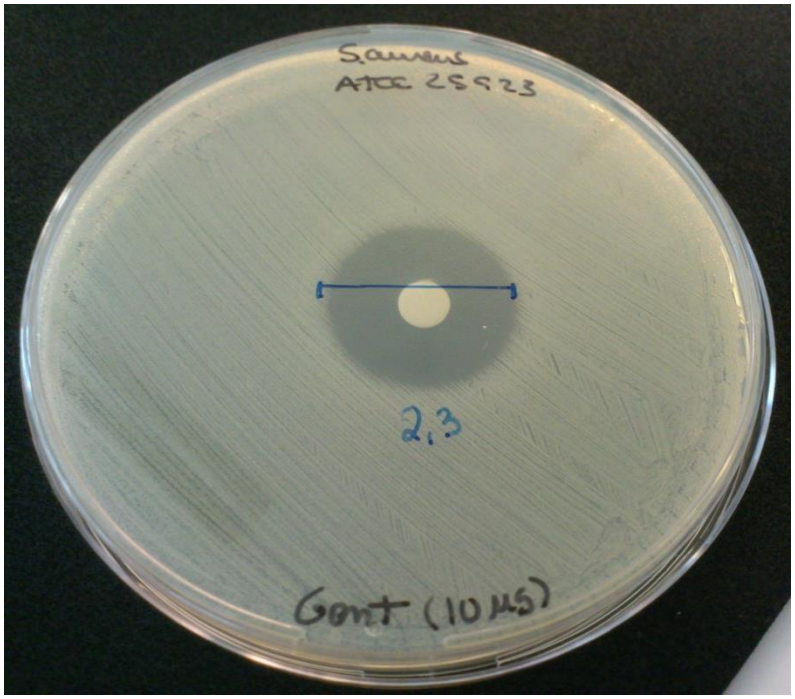

Figure 15| Antibiotic susceptibility profiles of *S. aureus* ATCC 25923 to gentamicin (10 µg). Growth in MHA medium for 18h, at 37 °C.