INTEGRATED MASTER IN BIOENGINEERING

Understanding the behavior of a drinking water biofilm model to chemical and mechanical stresses

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" Live as if you were to die tomorrow.

Learn as if you were to live forever."

Mahatma Gandhi

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Abstract

The development of biofilm in drinking water distribution systems (DWDS) became a concern because it can damage DWDS pipes. However the main problem is related with human health, since biofilms are the main responsible for the presence of microorganisms in drinking water (DW) and can be a reservoir for pathogens. Therefore, it is very important to understand the behavior and characteristics of biofilms in order to develop effective control strategies.

The aim of this dissertation was to understand the effects of a selected antimicrobial product, sodium hypochlorite (NaOCl), against single and dual species biofilm of two model bacteria, *Acinetobacter calcoaceticus* and *Stenotrophomonas maltophilia* and its influence in biofilm mechanical stability. Biofilms were formed on polyvinyl choride (PVC) using two distinct bioreactors: microtiter plates (24 h-aged biofilms) and a rotary cylinder reactor (7 d-aged biofilms). The biofilm behavior to chemical and mechanical stresses was assessed by exposing biofilms to different concentrations of NaOCl (0.1 ppm and MIC) and to different shear stresses (0.22, 0.72, 1.42 and 2.31 Pa). The action of NaOCl was also evaluated to planktonic and recently adhered cells through the analysis of bacterial surfaces (physicochemical characteristics, zeta potential, outer membrane proteins expression and motility) as well as by microscopic analysis of the attached cells number.

A. calcoaceticus have lower specific growth rate (μ= 0.0 47 ± 0.028 h^{-1}) than *S. maltophilia* (μ= 0.135 ± 0.001 h^{-1}) but both bacteria have similar surface characteristics, both are hydrophilic and have negative charged membrane. However the NaOCl have different effects in both, being *A. calcoaceticus* more susceptible to the disinfectant action. Both bacteria becomes less negatively charged when exposed to NaOCl, being also its outer membrane proteins and autoaggregation ability inhibited by NaOCl at MIC. However NaOCl treatment only affects the hydrophobicity and motility of *A. calcoaceticus*.

All these characteristics interfere in bacterial adhesion to the substratum and it was observed that *S. maltophilia* is more adherent to PVC than *A. calcoaceticus*, being also more resistant to the chemical treatment. The association of both bacteria confers more stability against NaOCl treatment in initial step of bacteria adherence to PVC, however

when biofilm is developed bacteria association appears not be an advantage against NaOCl exposition.

The pretreatment of 7 d-aged single and dual biofilms with NaOCl also causes alterations in its mechanical stability. The NaOCl treatment only promotes different *A. calcoaceticus* biofilm removal for different shear stresses being the overall removal similar. However the *S. maltophilia* becomes more resistant to mechanical stress when exposed to increasing concentrations of NaOCl, contrary to the dual species biofilm behavior.

Biofilms are resistant to both chemical and mechanical stresses, even if very high NaOCl concentrations and shear stress conditions were applied.

Resumo

A presença de biofilmes nos sistemas de distribuição de água potável tornou-se uma preocupação, podendo danificar as tubagens dos sistemas de distribuição. Contudo o principal problema causado pela sua presença está relacionado com a saúde humana, uma vez que são os principais responsáveis pela presença de microrganismos nos sistemas de distribuição de água. Os biofilmes podem constituir um reservatório de organismos patogénicos, protegendo-os dos fatores ambientais adversos. Por estas razões é de extrema importância o estudo das características dos biofilmes bem como formas de controlar o seu desenvolvimento.

O objetivo desta dissertação é compreender os efeitos de um produto antimicrobiano, o hipoclorito de sódio (NaOCl), contra biofilmes duplos e simples e a sua influência na estabilidade mecânica do biofilme, usando para tal duas bactérias modelo: *Acinetobacter calcoaceticus* e *Stenotrophomonas maltophilia.* Os biofilmes foram formados em superfícies de policloreto de vinil (PVC) usando dois reatores distintos: microplacas para formar biofilmes de 24 h e um reator de cilindros rotativos onde foram formados os biofilmes de 7 dias. O comportamento do biofilme face ao stress químico e mecânico foi avaliado através da exposição a diferentes concentrações de NaOCl (0.1 ppm e MIC) assim como pela exposição a diferentes tensões de corte (0.22, 0.72, 1.42 e 2.31 Pa). A ação do NaOCl foi avaliada quer para células planctónicas quer para células recentemente aderidas estudando as características da superfície bacteriana (características físico-químicas, proteínas da membrana externa e mecanismos de mobilidade) assim como por análise microscópica do número de células aderidas ao PVC.

A. calcoaceticus tem menor taxa de crescimento específico (μ= 0.0 47 ± 0.028 h^{-1}) do que *S. maltophilia* (μ= 0.135 ± 0.001 h^{-1}) embora ambas tenham características superficiais semelhantes, ambas são hidrofílicas e são carregadas negativamente. No entanto, o tratamento com NaOCl tem efeitos diferentes nas duas bactérias, sendo *A. calcoaceticus* a mais suscetível à ação do desinfetante. Ambas as bactérias tornam-se menos carregadas negativamente quando expostas a NaOCl, sendo também a expressão das proteínas da membrana externa e a sua capacidade de autoagregação inibidas na presença de NaOCl na

concentração mínima inibitória. Contudo o tratamento com NaOCl apenas afeta a hidrofobicidade e a mobilidade da *A. calcoaceticus.*

Todas estas caraterísticas interferem na adesão da bactéria a uma superfície, tendo sido observado que *S. maltophilia* tem maior capacidade de adesão ao PVC do que a *A. calcoaceticus,* sendo também mais resistente ao tratamento químico. A associação de ambas as bactérias confere mais estabilidade contra a ação do NaOCl em bactérias recentemente aderidas ao PVC. No entanto, quando os biofilmes estão mais desenvolvidos a associação das bactérias não parece ser vantajosa contra a ação do NaOCl.

O pré-tratamento de biofilmes de 7 dias com NaOCl causa alterações na estabilidade mecânica do biofilme. O tratamento com NaOCl apenas promove diferentes remoções do biofilme de *A. calcoaceticus* quando exposto a diferentes tensões de corte, contudo a remoção global é semelhante. Contrariamente, os biofilmes de *S. maltophilia* tornam-se mais resistentes ao stress mecânico quando são previamente expostos a concentrações crescentes de NaOCl. Também a estabilidade mecânica dos biofilmes duplos é afetada pelo tratamento químico, o tratamento com a concentração mínima inibitória de NaOCl enfraquece a estrutura do biofilme.

Os biofilmes mostraram-se resistentes quer ao tratamento químico quer ao tratamento mecânico, mesmo quando são usadas elevadas concentrações de NaOCl e elevadas tensões de corte.

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Glossary

ATP- Adenosine three phosphate

CBR- CDC biofilm reactor

CCR- Concentric cylinder reactor

ClO_2 - Chlorine dioxide

DBP- Disinfection-by-product

DLVO- Derjaguin- Landau- Verwey - Overbeek

DW - Drinking water

DWDS- Drinking water distribution systems

EPS- Extracellular polymeric substances

LBB- Luria-Bertani Broth

LPS- Lipopolysaccharides

MIC - Minimum inhibitory concentration

MRD- Modified Robbins Device

NaOCl - Sodium hypochlorite

OMP- Outer membrane protein

PBS- Phosphate- buffered saline

PEG- Poly (ethylene glycol)

PEO- Poly(ethylene oxide)

PVC- Polyvinyl chloride

PWG- Pennine Water Group

RIGP- Radiation -induced graft polymerization

RDR- Rotating disc reactor

TF- Traditional flushing

UDF- Unidirectional flushing

UV- Ultra- violet

XDLVO- Extended Derjaguin- Landau- Verwey - Overbeek

Chapter 1

1. Work Outline

1.1. Background and project presentation

There is a global concern for the water industry and for the regulatory agencies that all world population should have access to safe drinking water (DW). However, it does not happen, and there are many people without appropriate water to consume. It is essential to ensure the quality but also the quantity of DW. There is scarcity of water in some Earth areas and this problem, in addition to the presence of microorganisms, are responsible for an array of serious health problems. This is why DW is an important subject of study. However, the existence of DW distribution systems (DWDS) allows the water to be delivered to a wider population. However, the possible problems due to DWDS become a concern too (WHO 2011). The main problems reported in DWDS are the microbiological corrosion, biofilm formation and the occurrence and persistence of pathogenic organisms. Biofilms are considered as the main source of microorganisms in DWDS that are fed with treated water (Berry et al. 2006; Yu et al. 2010).

The DW treatment is a necessary process to ensure the public health security. DW is one of the most closely monitored resources. It is subjected to several treatment processes until it is ready to be distributed and consumed. However, after the distribution starts the treatment continues; DWDS has always a residual concentration of disinfectant in order to decrease the possible regrowth events (Deborde and von Gunten 2008). Also, mechanical strategies are used to keep the DWDS in safe conditions, like the pipe flushing. This is a method used in some countries that allows to remove the sludge and sediments from pipe walls, eliminates tastes and odours, reduces turbidity and restores the disinfectant residual concentration, affording a better control of microbial regrowth (Antoun et al. 1999; Friedman et al. 2002). The combination of disinfection and pipe flushing can be an efficient strategy to remove and control biofilms in DWDS.

1.2. Main objectives

The main objective of this work is to understand the effects of sodium hypochlorite (NaOCl) in *Acinetobacter calcoaceticus* and *Stenotrophomonas maltophilia* single and dual species biofilm removal from polyvinyl chloride (PVC) surface and its influence in biofilm mechanical stability. To do this work was used two bacteria isolated from DWDS, *A. calcoaceticus* and *S. maltophilia* and a rotary cylinder reactor as DWDS model. The effect of NaOCl was studied using two different concentrations, one is a residual concentration around the value present in DWDS (0.1 ppm) and the minimum inhibitory concentration (MIC) that was 300 ppm to *A. calcoaceticus* and 400 ppm to *S. maltophilia* and to the co-culture.

To evaluate the ability of these bacteria to adhere to PVC surface several methods were used, the surface charge of bacteria was measured through the measurement of Zeta potential, the hydrophobicity of cells also was determined as well as the prediction of theoretical adhesion through the measurement of contact angle applying the XDLVO theory. However are not only the physicochemical characteristics of cell that influence bacterial adhesion and biofilm formation, so also was studied the bacterial motility and their ability to auto and coaggregate. The effect of chemical treatment in all the studied characteristics also was performed, in order to understand the efficiency of different NaOCl concentrations in bacteria removal during initial stages of biofilm formation.

The biofilm susceptibility to NaOCl was studied to recently formed and steady state biofilms. The first mentioned biofilm were developed in microtiters and the NaOCl effect quantification was achieved through a microscopy assay to study the effect on cells removal from PVC surface. The steady-state biofilm were developed in a rotary cylinder reactor during 7 days at a constant shears stress of 0.09 Pa. The biofilm formed were exposed to NaOCl at different concentrations. Then it was studied the effect of this chemical treatment on biofilm mechanical vulnerability, by subjecting the biofilm in PVC cylinders to different shear stresses (0.22, 0.72, 1.42, 2.31 Pa).

1.3. Thesis organization

This work is organized in five chapters. Chapter 1 describes the main goals of this study and the outline of this dissertation.

Chapter 2 provides a brief literature review, focusing in the main problems of DWDS, namely the biofilm development and control. Information on selected strategies and devices used to understand biofilm development and control inside DWDS are also present in this chapter.

Chapter 3 describes the study of planktonic and adhered *A. calcoaceticus* and *S. maltophilia* characteristics and growth, being described the surface charge and hydrophobicity, the motility, the outer membrabe protein (OMP) expression and the ability to autoaggregate and coaggregate. The bacterial adhesion to PVC is previewed attending to the bacteria and PVC surface characteristics, and the real adherence to PVC coupons is also described, studying the influence of NaOCl on bacteria surface and in its removal from PVC.

Chapter 4 shows the behavior of 24 h- aged and 7 -d aged single and dual biofilms when exposed to NaOCl. The effects of NaOCl on 7 d- aged single and dual species biofilm mechanical stability also are described.

Chapter 5 describes the general conclusions from this work and provides some remarks for future work.

Chapter 2

2.Literature review

2.1. Drinking water needs and concerns

There is a global concern for the water industry and for the regulatory agencies that all world population have access to safe drinking water (DW). However, it does not happen, and there are many people without appropriate water to consume. It is essential to ensure the quality but also the quantity of DW. In fact, there is scarcity of water in some Earth areas and this problem is responsible for diverse serious health problems. This is why DW is an important subject of study (WHO 2011).

The WHO (2011) defines DW as water of enough quality to ensure that it can be used or consumed without risk of immediate or long-term harm. The consumption of chemical or biological contaminated water can be responsible for the development of a range of health-related problems in people, mainly those more susceptible (infants, children, elderly or immunocompromised people).

DW concept can be different in developing and developed countries. In developing countries the main concern is about microbial contamination that are responsible for 45% of all deaths being the chemical contamination insignificant. While in developed countries both problems constitute a concern (Gilbert 2012).

Chemical contamination can be divided in two different groups according to its origin: natural occurring inorganic chemicals (arsenic, radon and fluoride) and from anthropogenic activities, like lead, nitrate, pesticides (WHO 2011). More recently the emergent contaminants (human hormones, antibiotics, personal care products, endocrine disruptors and illicit drugs) seem to represent a significant part of the contaminants (De Gusseme et al. 2011). Chemical contamination can also be responsible for some human health problems: the ingestion of arsenic-contaminated DW can be responsible for dermal lesions, peripheral neuropathy, vascular disease and some kind of cancer (skin and lung); high fluoride intakes can cause serious problems in skeletal tissues; nitrite can react with

hemoglobin after the consumption of contaminated water blocking the oxygen transport in blood (methaemoglobinaemia); intake of lead-contaminated water can be responsible for problems in neurodevelopment, cardiovascular diseases, impaired renal function, hypertension, impaired fertility and adverse pregnancy outcomes (WHO 2011). Micropollutants, although in low concentrations, can also have some health impact, causing problems of resistance to antibiotics, endocrine disruption and also bioaccumulation in aquatic environments (De Gusseme et al. 2011).

The risks and impacts of chemical contaminants are a concern in developed countries. However, the risks from microbial pathogens remains ever present and waterborne diseases are one of the most important water-associated health problems (Beaudeau et al. 2008; Blasi et al. 2008). Waterborne diseases refer to any illness caused by the use of contaminated DW by opportunistic pathogens, particularly bacteria, protozoa, virus or helminths. Gastroentritis is the most known disease associated with waterborne outbreaks in developed countries; but other diseases exist and are a concern worldwide, particularly cholera, typhoid fever, meningitis, encephalitis, dysentery, hepatitis, legionellosis, pulmonary illness, poliomyelitis, leptospirosis, giardiasis and salmonellosis (Ashbolt 2004; WHO 2011).

The microbial contamination is not only a pre-DWDS problem, the quality of water can be changed during water treatment, storage and distribution (Bucheli-Witschel et al. 2012; Farkas et al. 2013). Therefore, the development of robust DWDS using adequate disinfection treatments is very important to reduce the incidence of waterborne diseases.

2.2. Microbiology in DWDS

DWDS are extreme environments where the availability of nutrients is very low and there is a constant residual concentration of disinfectant. Despite this, distribution systems contain a diverse microbial community of bacteria, protozoa, virus, algae and fungi. Included in these communities there are several genera that contain opportunistic pathogens, such as species of *Sphingomonas*, *Methylobacterium*, *Legionella*, *Pseudomonas*, *Stenotrophomonas* and non-tubercolous mycobacteria (Williams et al. 2004; Berry et al. 2006; Eichler et al. 2006; Poitelon et al. 2009; Revetta et al. 2010; Armbruster et al. 2012).

Most of bacteria present in the DWDS are attached to the pipe walls, only 5% of the DW bacterial community is suspended in the bulk phase (Flemming et al. 2002). However, the density of suspended bacteria increases between the treatment plant and the consumer's tap as a function of the disinfectant decay, hydraulic residence time, substrate uptake and the presence of corrosion deposits (Manuel et al. 2007). The biological stability of water is also changed according to other different factors in the DWDS. Those factors include the pipe material, flow velocity, temperature, existence of mixing and stagnation zones, sediments, the presence of biofilms or the intrusion of untreated water (Lautenschlager et al. 2013). The microbial presence in DWDS is responsible for many problems in DWDS, namely biofilm growth, nitrification, microbial mediated corrosion and the occurrence and persistence of pathogens (Berry et al. 2006). Bacterial growth within DWDS can also seriously affect hygienic and aesthetic quality of DW (Boe-Hansen et al. 2002).

Biofilm formation is one of the main concerns in DW quality management. Despite the presence of biofilms being a constant source of microorganisms, other problems are caused by the presence of this biological structure on the pipe walls that affect the DW quality: substances from the bulk water, including toxic chemicals, can be trapped into the biofilm matrix; biofilms can also work as a reservoir of pathogens and can promote microbial induced corrosion as is shown in the Figure 1 (Szewzyk et al. 2000; Beech and Sunner 2004; Castaneda and Benetton 2008; Teng et al. 2008; Douterelo et al. 2013).

Figure 1- Ductile iron pipe section from DWDS with a biofilm and high amounts of corrosion products (Chaves Simões and Simões 2013).

Microbial presence in DWDS is actually a global concern. Nowadays, it is very important to understand how microorganisms behave in DWDS in order to develop

efficient strategies to mitigate its growth and decrease its effects in the DWDS and in the public health (Lu et al. 2013).

2.2.1. Biofilms

Biofilm are a set of microorganisms embedded in a matrix of extracellular polymeric substances (EPS), which are secreted by them, and attached to a surface. Biofilms are a protective niche for the microorganisms (Fang et al. 2010). As biofilms occur usually in wet surfaces, their presence in DWDS is unavoidable.

Biofilm formation depends on several biotic and abiotic factor, namely environmental factors (temperature and pH), concentration of residual disinfectants, nature and concentration of nutrients, hydrodynamic conditions (flow rate, design of network and presence of dead ends), type and conservation of pipe materials, type and diversity of microorganisms and sediment accumulation (Deines et al. 2010; Jang et al. 2011; Yu et al. 2010). Bacteria seem to initiate biofilm development in specific environmental cues, such as nutrient availability (O'Toole et al. 2000). For example, biofilms will be formed most readily on surfaces that are rougher, hydrophobic and coated by surface conditioning films (Donlan 2002). Cell attachment can also be encouraged by an increase in flow velocity and by water temperature or nutrient concentration changes, if this factors do not exceed critical values (Vieira 1993; Simões et al. 2007b) So, the cell attachment to a surface and subsequent biofilm formation is a very complex process, with many variables affecting the process. This biological film is considered a stable point in a biological cycle that includes roughly four different steps (Figure 2): initiation, maturation, maintenance and dissolution (O'Toole et al. 2000).

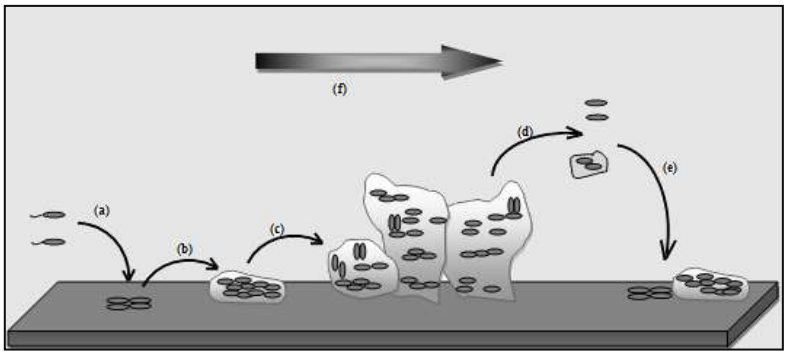

Figure 2- Biofilm formation, detachment and recolonization in DWDS. (a) -attachment, (b)- initiation, formation of colonies, starting of EPS production, (c)- biofilm maturation, (d)- biofilm dissolution, (e) - biofilm recolonization of DWDS pipes influenced by the water flow (f).

The amount of a biofilm in a given system after a certain period of time depends on a dynamic biofilm formation process, which has been defined as the balance between bacterial attachment from the planktonic phase, bacterial growth within the biofilm and biofilm detachment from the surface (Stoodley et al. 1999). When the balance is null, the biofilm is said to have reached a steady-state (Van der Kooij 1999). The biofilm detachment can be responsible for biofilm dispersion and formation/recolonization in other clean areas as is represented in the Figure 2 (Codony et al. 2005).

The control of biofilm formation becomes a priority in order to keep the water quality and preserve the human health, once biofilms can act as reservoir of pathogenic organisms and influence directly the bacterial density in the bulk phase due to detachment (Van Der Wende et al. 1989). Several studies have been done in order to understand how it is possible to control biofilm development. The influence of chemical treatment with biocides and disinfectants, mechanical treatment or physical pretreatment and also manipulation of the operational conditions are strategies studied to control the biofilm development in DWDS (Chandy and Angles 2001; Ollos et al. 2003; Ndiongue et al. 2005; Torvinen et al. 2007; Page et al. 2009; Douterelo et al. 2013).

Several works were done by playing with operational conditions in order to control the biofilm development. Ollos et al. (2003) evaluated several factors (biodegradable organic matter concentration, monochloramine and chlorine disinfection, pipe material, temperature) in biofilm development using an annular reactor as DWDS model. Temperature and flow velocity are factors studied using different DWDS models to understand how it can affect the biofilm formation in pipe walls (Pintar and Slawson 2003; Ndiongue et al. 2005; Torvinen et al. 2007; Lehtola et al. 2007; Douterelo et al. 2013). The nutrient concentration in the DW also can affect the biofilm development. Rubulis and Juhna (2007) attempted to control DW biofilm control by phosphorus removal. Tap water was subjected to coagulation and filtration processes in order to reduce phosphorus to the desired concentration. Chandy and Angles (2001) did a similar work, studying the influence of nutrients in biofilm growth by supplementing the tap water with potassium phosphate and sodium acetate.

The optimization of the chemical agents to disinfect DW is a typical research topic. However, most of the chemical treatments used to prevent biofilm development in DWDS pipes are based in chlorine addition. The disinfection of DW using free chlorine is widely used to achieve inactivation of a broad range of microorganism (Galal-Gorchev 1996). Many authors studied the chlorine effectiveness to inactivate the bulk water microorganisms (Corona-Vasquez et al. 2002; Goel and Bouwer 2004; Murphy et al. 2008; Shin and Sobsey 2008; Page et al. 2009); but it is also important to study the disinfection effects in biofilm microorganisms. In fact, the use of disinfectants can reduce the concentration of planktonic bacteria but have little to no effect on the concentration of biofilm bacteria has described Gagnon et al. (2005). These authors studied the relative efficiency of chlorite (0.25 and 0.1 $mg.L^{-1}$) and chlorine dioxide (0.5 and 0.25 $mg.L^{-1}$) on biofilm control. These disinfectants were used as a secondary treatment and the water fed to the reactor also was previously treated in a GAC (granular activated carbon) column. Chlorine dioxide promoted higher log reductions of attached heterotrophic bacteria with the use of high concentrations. The use of UV to control biofilm development also was studied in different works. However, it is only effective when is accompanied by chemical disinfection (Rand et al. 2007).

2.3. DWDS maintenance

Taking into account all the problems caused by the presence of microorganisms in DWDS in both planktonic and biofilm states, as described in Section 2.2.1, the DW treatment is a necessary process to ensure the public health security. DW is one of the most closely monitored resources that is subject to several treatment processes until being ready to be distributed and consumed (WHO 2011).

2.3.1. Disinfection

Water disinfection is a process used to kill or irreversibly inactivate microorganisms to ensure microbiologically safe water through DWDS. A chemical disinfection process that is based on unspecific oxidative processes to inactivate microorganisms is often used. The most used techniques to achieve an efficient disinfection of DWDS are chlorination,

chloramination or chlorine dioxide addition, ozone treatment and UV treatment (Chowdhury 2012).

Chlorination is a low cost process, easy to apply and able to inactivate a wide variety of pathogenic microorganisms. Therefore, this is the predominant disinfection method used in water treatment (Donnermair and Blatchley 2003). This disinfection method started to be used in large-scale at the beginning of the twenty century, becoming used worldwide few years later (Mir et al. 1997). DW disinfection process using chlorine can be done in two distinct points in the treatment process: pretreatment (induces a primary disinfection at the beginning of the treatment) and a post-treatment (to maintain a disinfectant residual in the distribution system, also called secondary disinfection). The use of chlorine as a post-treatment process plays an important role to control the microorganism regrowth (Deborde and von Gunten 2008).

The residual concentration of free chlorine used in the treatment plant should be less than 1 mg l^{-1} and nearer to 0.5 mg l^{-1} (WHO 2011). But this residual concentration appears not be enough to prevent the growth and development of microbial biofilms (Zhou et al. 2009). Chlorination can have a low activity in the microorganism inside the biofilms, but it can lead to a significant removal of the majority of planktonic bacteria (Bois et al. 1997; LeChevallier 1998). Xue et al. (2012) described that EPS are responsible for the resistance of microorganisms in biofilm to chlorine action. The presence of biofilm EPS protects cells by impairing chlorine diffusion and by the reaction of EPS with the disinfectant (Kumar and Anand 1998).

The chlorine action in cells viability was intensively studied. Firstly Baker (1926) theorized that chlorine destroys microorganisms by combining with proteins to form N-chloro compounds (LeChevallier 2004). Latter, Patton et al. (1972) found that chlorine has powerful effects on sulphydryl groups of proteins and converted several α-amino acids by oxidation. Chlorine can cause physiological damage to the bacterial cell membrane as cytochromes, iron-sulfur proteins and nucleotides are highly vulnerable to the chlorine oxidative effect. Therefore, respiration, glucose transport and ATP levels decrease in chlorine-treated bacteria (Camper and McFeters 1979; Virto et al. 2005; WHO 2011).

Zaske et al. (1980) found morphological changes in the membrane when observed chlorinated bacteria by electron microscopy.

Although being an efficient process, chlorination has some disadvantages, namely its dependency of water conditions, like pH and temperature. The concentration of chlorine used is also a critical aspect. High concentrations can cause organoleptic problems (strong odour and taste), and increase the production of disinfection by-products (DBP) that can be harmful to human health, like trihalomethanes and halogenic acids, which are carcinogenic. The use of chlorine in excess can also be responsible for the development of microbial resistance (Nieuwenhuijsen et al. 2000).

Other oxidant chlorine-based agents are also used in DW disinfection, such as chloramines and chlorine dioxide. Chloramines are less effective than free chlorine, produces less amount of DBPs and needs longer contact times or higher concentrations to achieve the desired disinfection level. However, it is more effective in biofilm penetration than chlorine (LeChevallier et al. 1988; Chandy and Angles 2001). The combination of chlorine and monochloramine promoted high disinfection levels with decreased amounts of DBPs (Momba and Kaleni 2002; Charrois and Hrudey 2007). Chlorine dioxide does not produce dangerous DBPs but produces chlorate and chlorite which can affect the water taste and odour. Its effectiveness in microbial disinfection is at similar or higher levels as chlorine. However, its use is not common (Huang et al. 1997; Schwartz et al. 2003; Chaves Simões and Simões 2013).

Non chlorine-based oxidants are also used efficiently in DW disinfection. Ozone is one of these oxidant agents which efficiently remove microorganisms. It is also effective for the inactivation of viruses and protozoa. Ozone does not affect the water taste and forms low amount of DBPs, but is more expensive than chlorine (Chaves Simões and Simões 2013).

The use of ultra-violet (UV) light is common to disinfect DW. UV energy (electromagnetic energy in the range 250-265 nm) unable the microorganisms to reproduce by altering their genetic material, being very effective against bacteria, viruses and protozoa cysts without producing DBPs (Liberti et al. 2003). UV irradiation cannot be used as a stand-alone disinfectant because it does not keep a residual concentration able to avoid

regrowth events (Hijnen et al. 2006). However, Schwartz et al. (2003) found that the use of UV treatment is not so effective in biological stability of water as chlorine dioxide treatment. The combination of UV as a primary disinfectant and free chlorine or monochloramine as secondary disinfectant shown the ability not only to prevent microbial regrowth but also to produce a synergistic disinfectant action (Ballester and Malley Jr 2004; Shang et al. 2007).

2.3.2. Flushing

Pipe flushing is an important process to maintain the water quality in DWDS being one of the least expensive used techniques. This consists in the replacement of contamined water by clean water through high velocity flow in pipes. This procedure can remove the sludge and sediments from pipe walls, eliminate tastes and odours, reduce turbidity and restores the disinfectant residual concentration, affording a better control of microbial regrowth (Antoun et al. 1999; Friedman et al. 2002). It is applied as a large-scale and periodic preventive measure or as response to localized water quality problems.

The main applied techniques of flushing are the traditional flushing (TF) and the unidirectional flushing (UDF). TF consists of opening one or more fire hydrants within a network area until the flushed water reaches the desired water criterion. TF must proceed from clean water source toward the network periphery (Antoun et al. 1999; Friedman 2004). UDF allows the pipe isolation through appropriate valve closure allowing pipe cleaning by unidirectional flush, opening at least one hydrant located at the termination of each pipe section. While TF allows the entire network flushing, UDF allows isolate a pipe area to clean unidirectionally. Less water is used in UDF but it is achieved higher flow velocity than TF causing less disturbance to consumers (Antoun et al. 1999; Friedman et al. 2002).

Douterelo et al. (2013) used an experimental DWDS to study the hydrodynamic influence in biofilm structure and composition through flushing application. Flushing altered the pipe-wall bacterial community structure but did not completely remove it from the surface, particularly under highly varied flow conditions, suggesting that under these conditions more compact biofilms were generated (Douterelo et al. 2013).

2.4. Devices used in DW biofilm studies

It is difficult to understand how biofilms are formed and preview their behavior and control inside of DWDS, so the development of appropriate devices to develop biofilm studies is much important.

It is essential that these devices are able to reproduce the real DWDS conditions. Some of these devices allow the study of formation, characterization, control of biofilms and also study the effect of some conditions *in situ*, it means that the devices can be put inside of DWDS (Calle et al. 2007; Henne et al. 2012) or inside of experimental plant devices (Percival et al. 1998; Lehtola et al. 2005; Srinivasan et al. 2008). In these cases there is an approximation of real systems, allowing the microbiological and metabolic characterization of biofilms and also allows the study of structural stability and biofilm control. Other devices were developed too and cannot be applied *in situ*, even they are also important to study biofilms. The use of reactors that simulate the DWDS conditions are used in order to increase the similarity of operation within the DWDS. The selection of the reactor system depends obviously on the goals to be achieved.

2.4.1. Bench top devices

Bench top devices were developed to study biofilms independently from the DWDS. These devices mimic the DWDS behavior, allowing to test different environmental conditions. They can be fed with tap water (previously treated or like it is distributed to the population) or with appropriate medium or enriched water.

The simplest device used to study DW biofilm development is the microtiter plates used to achieve different aims. Simões et al. (2010a) used this device to study the adhesion and biofilm formation on polysterene by drinking water-isolated bacteria. The same device was used by Simões et al. (2011b) to investigate the effects of metabolite molecules produced by bacteria isolated from DW on biofilm formation.

The concentric cylinder reactor (CCR) is a bench top device that was used to study DW biofilm by Rickard et al. (2004). The aim of the study was to describe the effects of different shear forces in DW biofilms formation and diversity. CCR is a reactor constituted by four rotating cylinder pipes and four stationary cylinder chambers. The chambers are feed with tap water and the volume inside the chambers is constantly controlled with the

help of external pumps, being the feeding ports different from the outlet and the sampling ports.

The flow cell reactor is often used to study DW biofilm formation. It is a semicircular duct with some coupons (only the upper face contacts with water) in its flat wall and the flow pass through the duct from the bottom to top. Usually, it is provided by a feed/fresh water reservoir and the temperature is controlled externally. The flow is recirculated and the sampling process do not stop the flow because outlet ports are located in the curved wall between two removal parts, allowing the deviation of the flow (Simões et al. 2006; Bragança et al. 2007; Manuel et al. 2007, 2010; Ginige et al. 2011; Simões et al. 2012).

The Propella® reactor consists in two concentric cylinders in which the propeller pushes the liquid down through the inner tube and then up through the annular section between the both cylinders. It is a perfectly mixed reactor and the fluid velocity, hydraulic residence time and the flow rate are controlled by the rotation speed of propeller. Coupons are usually located in the outer tube facilitating the sampling process and in some cases the removal of coupons do not change the flow conditions (Dailloux et al. 2003; Lehtola et al. 2006; Lehtola et al. 2007; Rubulis and Juhna 2007).

The continuous reactor probably mostly used in DW biofilm studies is the annular reactor. This reactor is used at least since 1990s (Morin and Camper 1997; Volk and LeChevallier 1999). It is a simple reactor that mimics the hydrodynamic behavior that biofilms are subjected in a DWDS (Batte et al. 2003; Keinänen-Toivola et al. 2006). This reactor, also known as Rototorque®, is constituted by two cylinders: the external is static and the internal is rotating, being its speed controlled by a motor (Morin and Camper 1997; Chandy and Angles 2001; Zhou et al. 2009; Fang et al. 2010; Hosni et al. 2011). Usually, the inner cylinder supports some coupons used to sample the biofilm. The rotation of the inner cylinder is controlled in order to define a desired shear stress.

The rotating disc reactor (RDR) consists in a tank with a rotating disc that is submerged in water. The disc holds several coupons disposed concentrically in the disc and, as happens with the CCR, the shear forces depends on the rotational speed and the diameter where coupons are inserted (Abe et al. 2011; Abe et al. 2012; Pelleïeux et al. 2012).

The CDC reactor, also known as CDC Biofilm Reactor (CBR), is sometimes used as model distribution system. It consists in a vessel with eight coupons holders supported by a ported lid, each holder contains usually 3 coupons. The lid with the holders is mounted in a vessel and the continuous mixing of the bulk fluid is provided by a baffled stir bar that is magnetically driven (Goeres et al. 2005; Morrow et al. 2008; Park and Hu 2010; Armbruster et al. 2012; Park et al. 2012).

A rotary cylinder reactor, although had not been used yet in DWDS biofilms studies, allows develop biofilms under controlled conditions. It was already used to study the effects of mechanical stress on biofilm control (Simões et al. 2005; Simões et al. 2009). This reactor consists in a tank with three rotary cylinders under constant rotation. The cylinders are submerged in the tank with a microbial suspension that operates as a chemostat. This device allows to form and study biofilms in a well-controlled, real-time and reproducible manner (Azeredo and Oliveira 2000).

2.4.2. In situ application devices

The use of *in situ* devices is important in DW biofilm studies, allowing to obtain results about the biofilm behavior in the real DWDS.

The Robbins device is one of the mostly used devices to study the biofilm behavior *in situ* (in real and plant scale DWDS). This device consists in a cylinder with several threaded holes. Some screws with coupons mounted on the front side are placed in these holes (Sly et al. 1990; Manz et al. 1993). The coupons are aligned parallel to the water flow and can be removed independently (Manz et al. 1993). Because the Robbins device promotes significant changes of water flow in slides, Nickel et al. (1985) developed a modified Robbins device (MRD) that consisted in a pipe with 25 spaced sampling ports attached to sampling plugs, flush with the inner surface, and, therefore, do not disturb the flow characteristics.

A recently developed device for *in situ* studies is the Pennine Water Group (PWG) coupon. This device is cut directly from the pipes and comprises two parts: a outer coupon and another that is inserted. The outer coupon retains the curvature of the pipe and fits precisely into a hole made in a removable and flanged identical pipe section. The coupon is fixed with a gasket to a section pipe. The inserted is engineered flat to allow microscope

analysis and is fitted inside the outer coupon in a way that the outer surface is in contact with water. This design has a maximum deviation from curvature of 0.064 mm, in the order of magnitude of the surface roughness coefficient used in hydraulic models (Deines et al. 2010). It is an accurate device and that allows direct insertion and close alignment with the internal pipe surface minimizing the distortion of boundary layer conditions that influence biofilm formation, such as boundary shear stress and turbulent driven exchange with the bulk water body (Douterelo et al. 2013).

The Bioprobe monitor was specifically designed to study biofilm growth within the pipe system. LeChevallier (1998) describes a pilot-scale DWDS (1.3 km) that had an experimental test station with 24 m, containing 3 test sections. At the beginning of each experimental section an instrument section to monitor the conditions and the biofilm development was located, called the Bioprobe monitor. The Bioprobe monitor consists of a pipe where a coupon holder (acetal) is inserted, being the coupon surface flushed with the pipe wall.

The aim to develop different devices to study biofilm formation and behavior is to try to mimic the conditions found in DWDS. When selecting a reactor, one can find that some are more appropriated for a specific study than others. For instance, to monitor biofilm growth it is better to use devices with several coupons to allow independent sampling. When comparing the use of bench top laboratorial reactors and *in situ* application devices, the first ones allow an easier control of the process conditions.

The advantages and limitations of a device can be related with the study of hydrodynamic conditions, with the sampling process, the temperature control, the type of material used, and the similarity with the real systems. The main advantages and limitations for the main devices presented are synthesized in the Table 1.

Table 1-Main advantages and limitations of each device referred above.

Reactors	Advantages	Limitations
Microtiter plates	Needs small space; temperature control; low shear stress control	Low similarity to DWDS, batch system; unable to study high shear stress conditions; volume limitation.
Robbins device	Can be applied to real DWDS - operational conditions very similar to reality.	The flow characteristics are changed with the presence of the coupons,; the operational conditions cannot be effectively controlled when used in real DWDS
Modified Robbins device	Can be applied to real DWDS, operational conditions very similar to reality; minimizes the changes in flow in the boundaries of coupons.	Difficulty to study different operational conditions
PWG coupon	Useful to be used use in pilot-scale DWDS; easy to take samples; do not change the flow conditions, curved structure as the DWDS pipes	Limitations in the control of operational conditions.
Bioprobe monitor	Allows to assess biofilm development *in situ*, coupled with monitor devices following the operational conditions; easy to take samples; changes in water flow are minimized	Limitation to control the operational conditions; Limited available information
CCR	Interesting to assess the role of hydrodynamic conditions on biofilms; allows to test different shear stress conditions at the same time.	Biofilm cannot be directly observed in microscope after coupon sampling; only one surface material can be tested; difficulty to take samples; temperature control depends on external devices.
CDC	Easy to take samples; allows the study of different materials at the same time; hydrodynamic conditions, controlled by an agitator.	The surface where biofilms are formed is flat; difficult control the shear stress; changes of the flow pattern in boundaries of the coupons
Propella	Easy to take samples; flow conditions controlled by a propeller, residence time controlled independently from the flowing process; flow conditions very similar to DWDS; allows the study of different materials at the same time;	Changes in the flow caused by coupons, temperature control achieved through the use of external systems
Rotating disc reactor	Study of different material coupons, control of operational conditions, hydrodynamic study, test different shear stress at same time, easy sampling.	The flow changes in the boundaries of the coupons; the biofilm is formed in a flat surface
Annular reactor	Allows the study of different materials at the same time; simulate the hydrodynamic conditions of real DWDS; easy to take samples; the surface of biofilm formation has a structure similar to the pipe.	The coupons change the flow patterns; the temperature control needs a jacket coupled to the reactor.
Flow cell reactor	Flow conditions similar to DWDS; easy to take sample; independent sampling at the desired time without changing or stopping the flow; allows the study of different materials at the same time;	Flow changed the by coupons; biofilms are formed on a flat surface; difficulty control the temperature inside the flow cell reactor
Rotating Cylinder reactor	Easy control of process conditions, mimics the DWDS	Biofilm cannot be directly observed in microscope after sampling

Chapter 3

3. Behavior of *A. calcoaceticus* and *S. maltophilia* planktonic and adhered cells in the presence of sodium hypochlorite

3.1.Introduction

Microbial adhesion will initiate the process of biofilm formation, where the microorganisms attach firmly to a surface (An and Friedman 1998). The development of a biofilm occurs in a sequential process that includes transport of microorganisms to the surface, initial (reversible or irreversible) adhesion, cell-cell communication, microcolonies formation, EPS production and biofilm maturation (Doyle 2000). There are different approaches used to describe bacteria-surface adhesion, namely the Derjaguin-Landai-Verwey-Overbeek (DLVO) theory, thermodynamic approaches and the extended DLVO (XDLVO) (Hori and Matsumoto 2010; Simões et al. 2010a). However, these approaches do not take into account some bacterial characteristics, such as the production of extracellular substances (EPS and lipopolysaccharides – LPS), the presence of appendages (fimbriae, pili, flagellum) and the ability of microorganisms to regulate differently their gene expression when they face distinct environmental conditions (Hori and Matsumoto 2010). An and Friedman (1998) described several factors that can affect bacterial adhesion to a surface, particularly:

1) bacterial physicochemical characteristics that varies according to bacterial species and is influenced by growth medium, bacterial age and structure (e.g. cell hydrophobicity and surface charge);
2) material surface physicochemical properties, like chemical composition, surface charge, hydrophobicity, roughness and texture;
3) environmental factors (temperature, pH, time exposure, bacterial concentration, chemical treatment and fluid flow conditions)

4) biological properties of the bacteria like LPS, EPS and other bacterial nanostructures.

Pavithra and Doble (2008) described adhesion as a two-phase process including an initial, instantaneous and reversible physicochemical phase and a time dependent and irreversible molecular and cellular phase. The initial phase is characterized by the movement of planktonic bacteria to the surface through the effect of physical forces. The physical interactions established during this phase can be divided in two groups, the long-range interactions (are nonspecific, act at distances superior than 150 nm and are responsible for the cell transport to the surface) and short-range interactions that are more important at closer proximity, acting at distances inferior than 3 nm. The second phase of adhesion is characterized by the predominance of molecular and cellular reactions between bacterial surface structures and substratum surfaces, being this an irreversible process. Ishii et al. (2004) found that *Acinetobacter sp*. strain Tol 5 has the ability to adhere in only one step through its appendages: anchor and peritrichate fibril. The anchor extends straight to the substratum at distances of several hundred nanometers whereas the peritrichate fibril attaches to the substratum in multiple places, fixing the cell at much shorter distances.

Some strategies are reported to control bacterial adhesion. The use of antimicrobial agents is the most applied. The surface modification can also be effective to avoid bacterial adhesion and some anti-adhesive surfaces have been reported, such as poly ethylene oxide (PEO), poly ethylene glycol (PEG) and radiation induced graft polymerization (RIGP) that alter the substratum surface (Hori and Matsumoto 2010).

The behavior of cells in the earlier stages of biofilm formation and its relationship with the biofilm development is an important issue to be studied in order to understand how a biofilm is developed and how is its susceptibility to removal strategies. However there is a lack of information regarding this issue.

The aim of this study was to characterize the behavior of *Acinetobacter calcoaceticus* and *Stenotrophomonas maltophilia* in both planktonic and sessile states in the presence and absence of NaOCl.

3.2.Materials and methods

3.2.1. Microorganisms and culture conditions

A. calcoaceticus and *S. maltophilia* were isolated from a DWDS and identified by 16S rRNA gene sequencing as described previously (Simões et al. 2007a).

Bacterial cells were grown overnight in batch culture using a nutrient concentrated medium (glucose 5 g/L, peptone 2.5 g/L, yeast extract 1.25 g/L and 0.2 M phosphate buffer at pH 7) at room temperature (23 ± 3 ºC) and under agitation (120 rpm) in an orbital incubator (New Brunswick Scientific, I26, USA). Glucose and yeast extract were obtained from Merck and peptone from Liofilchem.

3.2.2. Bacteria preparation

Bacteria were harvested by centrifugation (Eppendorf centrifuge 5810R) during 12 minutes at 3777 g, washed two times in phosphate-buffered saline (0.2 M PBS, pH 7) and resuspended in the same buffer. The optical density at 610 nm was adjusted to 0.4. These cells were used for free energy of adhesion and hydrophobicity assays and for adhesion to polyvinyl chloride (PVC) coupons.

3.2.3. Planktonic growth curves

The growth curves were obtained for *S. maltophilia* and *A. calcoaceticus* single and dual species cultures through the monitoring of the optical density at 610 nm, using a spectrophotometer (VWR V-1200), according to Simões et al. (2008b). This procedure was performed in duplicate.

3.2.4. Minimum inhibitory concentration determination

The minimum inhibitory concentration (MIC) of NaOCl (Sigma) for *S. maltophilia* and for *A. calcoaceticus* was determined by the broth microdilution method according to McBain et al. (2004). A pre-culture grown as described in Section 3.2.2 was used as inoculum in susceptibility tests, after adjusting its optical density to 0.1 (610 nm). The test was performed in 96-well microtiter plates (Orange Scientific). Several solutions of NaOCl (0.1 - 5000 ppm) were prepared through a stock solution at 10% (v/v). A volume of 20 µL of each solution was added to each well containing 180 µL of cell cultures. The optical

density was measured, in a microtiter plate reader at 610 nm (SpectraMax M2E, Molecular Devices), before and after 24 h of incubation at room temperature (23 ± 3 ºC) and 120 rpm.

The MIC corresponded to the lowest concentration of NaOCl at which no growth was found.

3.2.5. Free energy of adhesion and surface hydrophobicity

The free energy of adhesion between the bacterial cells and PVC surfaces was assessed according to the procedure described by Simões et al. (2007a). The cell suspensions previously prepared as described in Section 3.2.2. were filtered (50 mL) through a membrane (0.45 µm, Whatman) in order to achieve an homogeneous layer of cells. To test the NaOCl effects on surface hydrophobicity, the cell suspensions were exposed to this product during 30 minutes to 0.1 ppm and to the MIC (400 ppm to *S. maltophilia* and 300 ppm to *A. calcoaceticus*). The surface tension of bacterial surfaces were determined using the sessile drop contact angle method according to Simões et al. (2010a). The measurements were carried out at room temperature (23 ± 3 ºC) using three different liquids, water, α- bromonaphtalene and formamide (Sigma). Determination of contact angles was performed automatically using a model OCA 15 Plus (DataPhysics, Germany) video based optical contact angle measure instrument, allowing image acquisition and data analysis. Contact angle measurements (at least 20 determinations for each liquid and for each microorganism and material) were performed. The reference liquids surface tension components were obtained from literature (Janczuk et al. 1993).

Cells and PVC hydrophobicity were assessed using the approach of van Oss et al. (1987, 1988, 1989). In this approach, the degree of hydrophobicity of a given material (i) is expressed as the free energy of interaction between two entities of that material when immersed in water (w)- ΔG_{iwi}. If the interaction between the two entities is stronger than the interaction of each entity with water, $\Delta G_{iwi}^{TOT} < 0$ mJ/m^2, it means that the material is hydrophobic, but if $\Delta G_{iwi}^{TOT} > 0$ the material is considered hydrophilic. ΔG_{iwi} can be calculated through the surface tension components of interacting entities according to Equation 1:

$$\Delta G_{iwi}^{TOT} = -2\left(\sqrt{\gamma_i^{Lw}} - \sqrt{\gamma_w^{Lw}}\right)^2 + 4\left(\sqrt{\gamma_i^+\gamma_w^-} + \sqrt{\gamma_i^-\gamma_w^+} - \sqrt{\gamma_i^+\gamma_i^-} - \sqrt{\gamma_w^+\gamma_w^-}\right) \quad \text{(Eq. 1)}$$

where,

γ^{Lw} is the Lifshitz-van der Waals component of the surface free energy

γ^{+} represents the electron acceptor parameter;

γ^{-} represents the electron donor parameter;

γ^{AB} is the Lewis acid-base component, with $\gamma^{AB} = 2 \times \sqrt{\gamma^{+}\gamma^{-}}$

The surface tension components of a surface (bacteria or substratum) are obtained by measuring the contact angle of the referred three pure liquids with well known surface tension components and by simultaneous resolution of three equations of the form of Equation 2.

$$(1 + cos\theta)\gamma_i^{TOT} = 2\left(\sqrt{\gamma_s^{Lw}\gamma_i^{Lw}} + \sqrt{\gamma_s^{+}\gamma_i^{-}} + \sqrt{\gamma_s^{-}\gamma_i^{+}}\right) \quad \text{(Eq. 2)}$$

where θ is the contact angle and $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$.

The interaction between substrate i and v that are immersed or dissolved in water is represented by the total interaction energy (ΔG_{iwv}^{TOT}) and is expressed as shown by Equation 3.

$$\Delta G_{iwv}^{TOT} = \gamma_{iv}^{Lw} - \gamma_{iw}^{Lw} - \gamma_{vw}^{Lw} + 2\left[\sqrt{\gamma_w^{+}}\left(\sqrt{\gamma_i^{-}} + \sqrt{\gamma_v^{-}} - \sqrt{\gamma_w^{-}}\right) + \sqrt{\gamma_w^{-}}\left(\sqrt{\gamma_i^{+}} + \sqrt{\gamma_v^{+}} - \sqrt{\gamma_w^{+}}\right) - \sqrt{\gamma_i^{+}\gamma_v^{-}} - \sqrt{\gamma_i^{-}\gamma_v^{+}}\right] \quad \text{(Eq. 3)}$$

Thermodynamically, if $\Delta G_{iwv}^{TOT} < 0$ mJ/m^2 adhesion is favorable, on the other hand, if $\Delta G_{iwv}^{TOT} > 0$ adhesion is not expected to occur.

3.2.6.Adhesion assay

Adhesion tests were performed with *S. maltophilia*, *A. calcoaceticus* and their mixture, using PVC as adhesion surface. Also, the effect of NaOCl on the adhered cells was studied. Bacteria were prepared as described in Section 3.2.2. The PVC coupons (1 cm × 1 cm) were washed with detergent, ethanol and then sterile water. Afterwards, the coupons were inserted in a 48-wells microtiter plates (Nunc, Denmark) and 1.2 mL of each cell

suspension was added to each well. To test the adhesion of both bacteria simultaneously 600 µL of each culture was added.

The adhesion to PVC coupons was allowed to occur for 2 h at 23 ± 3 ºC in an incubator (New Brunswick Scientific, I26, USA) at 120 rpm. The negative control was obtained placing the PVC coupons in PBS without bacterial cells.

The experiment was performed in triplicate and at the end of the assay each coupon was carefully washed with PBS and was observed using an epifluorescence microscope Leica DM LB2 (Leica Microsystems, Germany) after staining with 400 µL of 4', 6-Diamidino-2-Phenylindole (DAPI, Sigma) at 0.5 µg/mL, according to Simões et al. (2007c). The stained coupon was then incubated during 10 minutes in dark. The optical filter combination for optimal viewing of stained preparations consisted of a 359 nm excitation filter in combination with a 461 nm emission filter. Twenty micrographs per coupon were obtained using a microscope camera (AxioCam HRC, Carl Zeiss).

The same experiment was performed testing the effects of NaOCl at 0.1 ppm and at the MIC (400 ppm for *S. maltophilia* and the consortium, and 300 ppm for *A. calcoaceticus*). After 2 h of incubation, the PVC coupons in the microtiter plate were exposed to NaOCl for 30 minutes. Afterwards, the number of cells attached to PVC was assessed by DAPI staining, as referred previously.

3.2.7.Zeta potential measurement

The zeta potential of PVC and cell suspensions, before and after contact with NaOCl (0.1 and MIC value - 400 ppm to *S. maltophilia*, 300 ppm to *A. calcoaceticus*), was determined using a Nano Zetasizer (Malvern Instruments, UK). Cell suspensions without biocide were used as controls. The zeta potential was measured by applying an electric field across the bacterial suspensions. Bacteria in the aqueous dispersion with non-zero zeta potential migrated towards the electrode of opposite charge, with a velocity proportional to the magnitude of the zeta potential. The experiments were done in triplicate.

The bacterial cultures were previously prepared similarly as the described in Section 3.2.3, however, PBS was not used to wash the pellet. Cells were washed twice with sterile distillated water. PVC was used as powder to allow the assessment of its zeta potential.

3.2.8. Motility

Overnight cultures grown in Luria–Bertani broth (LBB) (Merck) were used to characterize bacterial motility. Fifteen µl of these cultures were applied in the center of plates containing 1% tryptone (Merck), 0.25% NaCl (Merck), and 0.3%, 0.7% or 1.5% (w/v) agar (Merck) for swimming/colony spreading, swarming and twitching motilities, respectively (Butler et al. 2010; Stickland et al. 2010). The use of different concentrations of agar enables the characterization of different types of bacterial motility, once the medium porosity is directly related to the concentration of agar. So, various levels of bacterial diffusion could be selected. NaOCl at 0.1 ppm and the MIC were incorporated into the growth medium in order to determine if its concentration affects bacterial motility. Then, the plates were incubated at 23 ± 3 ºC and the diameter (mm) of the bacterial motility halos were measured at 24, 48 and 72 h of incubation. Three plates were used to evaluate the motility of each bacterium.

3.2.9.Coaggregation assays

The coaggregation assay was performed using *S. maltophilia* and *A. calcoaceticus* in stationary phase of growth, cells grown as described in Section 3.2.2 were harvested by centrifugation (20 min at 3777 g) and washed three times and resuspended with sterile tap water.

The coaggregation and autoaggregation abilities of the tested bacteria were assessed over time (0, 2, 24 and 48 h), according to Simões et al. (2008a). The effects of NaOCl (at 0.1 ppm and the MIC) was also assessed on the bacterial coaggregation and autoaggregation abilities. The NaOCl solution was added to cell suspensions and after 30 minutes of contact the first observation was done, being this considered the result at initial time (0 h).

It was used a visual coaggregation assay according to Cisar et al. (1979). Each bacterial suspension at an optical density of 1.5 (610 nm) was mixed together at equal volumes (1 mL of each) in rolled glass tubes at 23 ± 3 ºC. Control tubes were also used as autoaggregation assay, putting only 2 mL of one bacterium. Afterwards, each mixture was vortexed for 10 s and then each tube was rolled gently for 30 s.

If specific cell-to-cell recognition occurs, cells coaggregate and settle down. The scoring criteria were: 0- no visible coaggregates in the cell suspension; 1- very small uniform coaggregates in a turbid suspension; 2- easily visible small coaggregates in a turbid suspension; 3- clearly visible coaggregates which settle, leaving a clear supernatant; and 4 - very large flocs of coaggregates that settle almost instantaneously, leaving a clear supernatant. The autoaggregation tubes were also analyzed by the same criteria.

Coaggregation was considered to be present if the score in the tubes with the bacterial mixture were higher than the score of the autoaggregtaion assay.

3.2.10.Outer membrane proteins (OMP) extraction

The OMP was isolated according to the method described by Winder et al. (2000). Cells were harvested by centrifugation (15 min at 3777 *g*) and then were washed three times with PBS and also resuspended in PBS. Cells suspension with an optical density of 0.4 (610 nm) were exposed to NaOCl (0.1 ppm and MIC) for 30 minutes. The control experiment was done without NaOCl addition. Afterwards, each suspension was centrifuged and the pellet was suspended in 25 mM Tris and 1 mM $MgCl_2$ (Merck) buffer (pH 7.4). Sonication was used to promote cell lysis, each sample were sonicated till form foam (5 × 20s). After that, solutions was centrifuged (7000 g, 10 min, 4 ºC in a Beckman Avanti J25 centrifuge) to remove non-lysed cells. The supernatant was collected and the inner membrane protein were solubilized adding sarcosine (Sigma) 0.2 % (w/v) and was kept on ice during 20 minutes, being then added 25 mL of 25 mM Tris and 1 mM $MgCl_2$ buffer (pH 7.4). The solution was then centrifuged (13 000 g, 1 h, 4 ºC) and washed with non-sterile distillated water and centrifuged again in the same conditions (13 000 g, 1 h, 4 ºC). The pellet was resuspended in 300 µL of 25 mM Tris-HCl buffer (pH 7.4) and stored at -20 ºC until be used.

3.2.11.SDS-Page

The protein content in each sample was determined through the Bradford test in a 96 well microtiter plate (Orange Scientific) in order to insert the same quantity of protein in each well of the gel (adding 15 µL of protein solution at 200 µg/mL in each well).

The OMP samples obtained were subjected to SDS-PAGE with 12% (w/v) acrylamide (Bio-rad). Electrophoresis was performed at a constant current of 10 mA. After electrophoresis, the gels were Coomassie blue (Bio-rad) stained for protein profile detection (Laemmli 1970).

3.2.12. Statistical analysis

Data was analyzed using One Way ANOVA from the statistical software SPSS 20.0 (Statistical Package for the Social Sciences) assuming a significance level for the separation set at $p < 0.05$.

3.3.Results and discussion

3.3.1.Bacteria growth rate

A. calcoaceticus and *S. maltophilia* are Gram negative bacteria usually present in DWDS (Simões et al. 2010b, 2011b). *A. calcoaceticus* is an opportunistic pathogen, however, it appears to have little invasive power which depends on a pre-existing break in the normal body defenses (Pal and Kale 1981). *S. maltophilia* is also an emerging nosocomial pathogen associated with several infectious diseases and opportunistic infections especially in immunocompromised patients. This bacterium is able to adhere avidly to medical implants and catheters forming biofilms (De Oliveira-Garcia et al. 2003). In addition to their pathogenicity, *S. maltophilia* is intrinsically resistant to multiple antibiotics and disinfectants and clinical isolates often display a high level of multidrug resistance (Zhang et al. 2000).

Table 2 shows the specific and maximum growth rate as well as the doubling time for *A. calcoaceticus* and *S. maltophilia* single and dual cultures. *A. calcoaceticus* exhibited a slower specific growth rate as well as slower maximum growth rate than *S. maltophilia* ($p<0.05$). The values obtained for the consortium had an intermediate value between the two single cultures being the growth of the consortium influenced by both bacteria. The curve shapes in Figure 3 also confirms that growth is influenced by both bacteria when the bacteria were co-cultured.

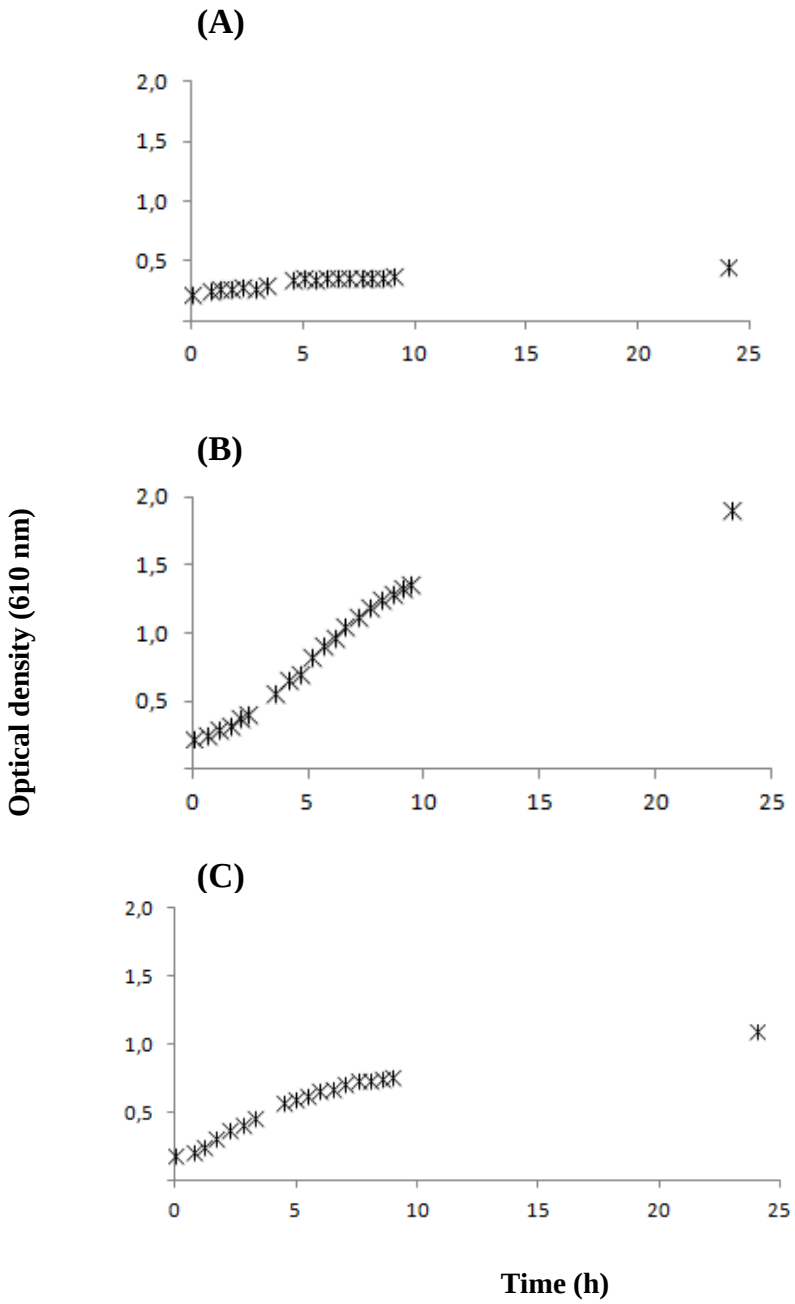

Figure 3- Growth curve of *A. calcoaceticus* (A), *S. maltophilia* (B) and their co-culture (C) at 23 ± 3 ºC and 120 rpm.

The analysis of the growth profiles (Figure 3) shows that *A. calcoaceticus* has a shorter exponential phase, starting the stationary phase 4.5 h after incubation, while *S.*

maltophilia achieved its stationary phase later (10 h after incubation). Concerning the co-culture growth profile, it appears to achieve the stationary phase 7 h after.

Table 2- Specific growth parameter of *S. maltophilia, A. calcoaceticus* and their co-culture at 23 ± 3 ºC, 120 rpm, (μ is the specific growth rate, μ_{max} is the maximum growth rate and t_d is the doubling time).

	μ (h^{-1})	**t_d (h)**	**μ_{max} (h^{-1})**
A. calcoaceticus	0.047 ± 0.028	17.9 ± 3.3	0.104 ± 0.044
S. maltophilia	0.135 ± 0.001	5.8 ± 0.4	0.246 ±0.013
Consortium	0.082 ± 0.003	7.5 ± 1.1	0.271 ± 0.001

When comparing these results with those described in literature, the first ones show lower specific growth rates for both bacteria. Cutter and Stroot (2008) presented a value of *A. calcoaceticus* growth rate of 0.381 h^{-1} when bacteria grew at 25 ºC in nutrient broth, at 250 rpm, while Dunne et al. (2000) found a specific growth rate of *S. maltophilia* between 1.09-1.28 h^{-1} when it was grown in LB liquid at 28 ºC. The bacteria used in this work were environmental bacteria isolated from DWDS, so they are stressed due to the continuous exposure to adverse conditions, such as NaOCl and shear stress. This fact can explain the lower growth rate achieved for both *A. calcoaceticus* and *S. maltophilia*. However, the bacterial growth rate can also be influenced by experimental conditions such as medium used, temperature and velocity of agitation that were different in the studies mentioned above, from the present work.

The effect of NaOCl in bacterial suspension growth was studied giving the MIC value for each bacterium. During this study it was possible to observe a decrease of bacterial growth even at residual concentrations of NaOCl (results not shown). However, the MIC were of 400 ppm and 300 ppm for *S. maltophilia* and *A. calcoaceticus*, respectively. This result shows that *S. maltophilia* can be more resistant to NaOCl than *A. calcoaceticus* (Table 3).

Table 3-Minimum inhibitory concentration of NaOCl to *A. calcoaceticus* and *S. maltophilia*.

	MIC (ppm)
A. calcoaceticus	300
S. maltophilia	400

3.3.2. Physicochemical and charge characterization of bacterial surfaces

Bacterial adhesion can be influenced by the surface physicochemical properties of both bacteria and substratum. Therefore, *A. calcoaceticus*, *S. maltophilia* and PVC were characterized in terms of surface properties, namely surface charge and hydrophobicity.

The charge of the bacterial surface was determined using a zeta potential analysis which measures the mobility of cells in the presence of an electrical field. Table 4 presents the zeta potential for *A. calcoaceticus* and *S. maltophilia* subjected to different concentrations of NaOCl. Both species have a negative charged membrane being *A. calcoaceticus* less negatively charged than *S. maltophilia* ($p<0.05$). The negative charge of the bacterial surface may occur due to the presence of anionic groups in their membranes, like carboxylic and phosphate groups (Ahimou et al. 2002).

Similar values of zeta potential were described by other authors. Mei et al. (1991) used two different strains of *A. calcoaceticus* and studied the effects of pH on its zeta potential. To a neutral pH they found a zeta potential around -20 mV to a hydrophobic strain (RAG1) and around - 33 mV to a hydrophilic strain (MR-481). *S. maltophilia* zeta potential was analyzed by van Merode et al. (2007) after being isolated from clogged biliary stents. The values obtained ranged between -14 ± 1 and -24 ± 1 mV.

The addition of NaOCl to cell suspensions caused the increase of zeta potential, meaning that the membrane becomes less negatively charged ($p<0.05$). This result was not expected since NaOCl is an electrically neutral compound. Nevertheless, NaOCl is able to cause significant changes in the bacterial surface charge.

Table 4- Zeta potential values to *A. calcoaceticus* and *S. maltophilia* after exposure to different concentrations of NaOCl. The MIC is 300 ppm to *A. calcoaceticus* and 400 ppm to *S. maltophilia*.

[NaOCl] (ppm)	**Zeta potential (mV)**	
	A. calcoaceticus	***S. maltophilia***
0	-28.0 ± 0.6	-30.7 ± 1.4
0.1	-24.0 ± 1.1	-20.5 ± 0.7
MIC	-22.0 ± 0.4	-14.6 ± 1.4

To predict the possibility of bacteria to approach to the PVC surface, its zeta potential was also measured being this material positively charged (11.3 ± 0.6 mV). Attending only to the charge of surfaces, bacteria should not be repelled by PVC as both surfaces have opposite charges.

Regarding surface hydrophobicity of both bacteria, it was evaluated using an approach proposed by van Oss (1997), which allows the assessment of the absolute degree of hydrophobicity of bacteria in comparison with their interaction with water. Table 5 shows the surface tension and hydrophobicity characteristics of *A. calcoaceticus*, *S. maltophilia*, and their values when exposed to different concentrations of NaOCl.

Table 5- Surface tension parameters (γ_{lw}- Lifshitz- van der Waals component ; γ_i^+- electron acceptor parameter ; γ_i^-- electron donor parameter; γ_{AB} - Lewis acid-base component) and hydrophobicity (ΔG_{iwi}^{TOT}) characteristics of *A. calcoaceticus* and *S. maltophilia* after being exposed during 30 minutes to different concentration of NaOCl.

Bacteria	**[NaOCl] (ppm)**	**Surface tension parameters (mJ/cm^2)**				**Hydrophobicity (mJ/m^2)**
		γ_{lw}	γ_i^+	γ_i^-	γ_{AB}	ΔG_{iwi}^{TOT}
A. calcoaceticus	**0**	25.6 ± 0.9	5.2 ± 0.8	44.4 ± 2.1	30.1 ± 1.8	17.7 ±2.8
	0.1	35.9 ± 1.3	1.6 ± 0.4	52.2 ± 3.1	18.4 ± 1.7	29.4±4.0
	300	33.3 ± 1.5	0. 0 ± 0.0	77.1 ± 7.8	0.0 ± 0.0	72.7 ± 9.0
S. maltophilia	**0**	29.8 ± 5.2	5.4 ± 1.3	53.3 ± 2.3	29.2 ± 6.2	27.1 ± 5.4
	0.1	36.4 ± 1.2	1.1 ± 0.8	47.7 ± 7.3	15.5 ± 3.4	29.2 ± 6.4
	400	31.0 ± 1.1	1.8 ± 0.4	49.5 ± 3.6	19.7 ± 2.7	27.1 ± 5.4

According to the classification described by van Oss (1997) *A. calcoaceticus* is an hydrophilic bacteria ($\Delta G_{iwi}^{TOT}>0$ mJ/m^2). The exposure to NaOCl at a residual concentration (0.1 ppm) is responsible by a small increase of total interaction energy (from -17.7 ± 2.8 to 29.4 ± 4.0 mJ/m^2) becoming the bacteria surface more hydrophilic ($p<0.05$). The exposure of *A. calcoaceticus* to NaOCl at MIC (300 ppm) causes a sudden increase of ΔG_{iwi}^{TOT} ($p<0.05$) becoming the cell suspension highly hydrophilic (72.7 ± 9.0 mJ/m^2). The electron donor parameter of *A. calcoaceticus* increases with the use of high concentrations of NaOCl (from 44.4 ± 2.1 to 77.1 ± 7.8 mJ/cm^2). The acceptance of electrons suffer an opposite effect, decreasing when it is used higher biocide concentration, losing all its ability to accept electrons when exposed to the MIC during 30 minutes (0 mJ/m^2).

S. maltophilia is also an hydrophilic bacteria (ΔG_{iwi}^{TOT} of 27.1 ± 5.4 mJ/m^2) and this characteristics is not affected by the application of NaOCl at 0.1 ppm and MIC ($p>0.05$). After all the treatments with NaOCl, *S. maltophilia* showed to have both donor and acceptor electron abilities, and its values were not affected with the increase of NaOCl concentration ($p>0.05$).

Simões et al. (2007a, 2010a) described these bacteria as hydrophilic. In these studies surface properties of *A. calcoaceticus* isolated from DWDS were evaluated, and two different results of surface hydrophobicity were found: 30 and 7 mJ/m^2. Simões et al. (2007a) also described the surface properties for two strains of *S. maltophilia* isolated from DWDS, being the hydrophobicity 14.7 and 15.4 mJ/m^2. The differences between these results can be explained based on the variation caused by bacteria age and growth medium between the studies (An and Friedman, 1998).

Data about bacteria and support material hydrophobicity allows the prediction of bacterial adhesion. As *A. calcoaceticus* and *S. maltophilia* are classified as hydrophilic bacteria and PVC is an hydrophobic material ($\Delta G_{iwi}^{TOT}<0$ as presented in the Table A.1 at Appendix A), the adhesion of this bacteria to PVC surfaces appear to be not favorable due to the repulsion between the hydrophilic surface of bacteria and the hydrophobic phase of PVC (Simões et al. 2007a).

3.3.3. Bacterial motility

Bacterial adhesion to a substratum is not only affected by bacteria physicochemical surface properties. Also, bacterial motility may influence biofilm formation and development, helping cells to reach the most favorable environments and to compete with other microorganisms in response to external conditions (Fenchel 2002; Stocker et al. 2008; Maes et al. 2013). Cell motility is characterized by the presence of structures like flagella, pili or fimbriae that play an important role in microorganisms initial adhesion to a surface and consequent biofilm formation. These structures are able to overcome the repulsive forces associated with the substratum and can contribute to the overall surface hydrophobicity (O'Toole and Kolter 1998; Donlan 2002; Mandlik et al. 2008).

Several types of bacterial motility were already defined, such as swimming, swarming and twitching. Swimming and swarming are dependent on the flagella presence and can contribute to the virulence of pathogens, helping the bacteria to adhere and to form biofilms on both biotic or abiotic surfaces. Swarming has influence in the initial interaction with surfaces, but it also allows the cells to move along the surfaces. Therefore, swarming has an important role in the early stages of biofilm formation (Henrichsen 1972). Twitching motility is associated with the presence of type IV pili and is proposed to facilitate movement along the surface. Twitching also contributes to the formation of microcolonies within a developing biofilm (Pratt and Kolter 1998).

Table 6 presents the results obtained to diameter of motility halos for *A. calcoaceticus* and *S. maltophilia* exposed to different concentrations of NaOCl. *A. calcoaceticus* showed a halo increase over time in swimming ($p<0.05$) and twitching ($p<0.05$) motilities when not exposed to NaOCl. The swimming motility of *A. calcoaceticus* is more significant than twitching and *A. calcoaceticus* appears not to have swarming motility. The diameter of swimming halos decreased with increasing concentrations of NaOCl ($p<0.05$) so the ability of *A. calcoaceticus* to attach to the surface and to overcome repulsive forces decreases when exposed to NaOCl. The exposure to NaOCl also reduces the twitching of *A. calcoaceticus*, despite the twitching motility decrease was similar for both NaOCl concentrations tested ($p>0.05$).

S. maltophilia had swimming, swarming and twitching motilities. The effects of NaOCl appear to be smaller than the observed for the *A. calcoaceticus* assay ($p<0.05$). The effect of NaOCl on swimming, swarming and twitching was not significant even when NaOCl was applied at the MIC. Therefore, it can be concluded that the exposure of *S. maltophilia* to NaOCl does not affect adhesion mediated by motility.

Some literature data corroborates the values achieved in this work. Henrichsen and Blom (1975) found that *A. calcoaceticus* is able to move trough twitching motility, while the tests done by Simões et al. (2007b) described also that this bacteria is able to move through swimming motility. Pompilio et al. (2008) described that most of *S. maltophilia* tested strains are able to move by swimming and twitching motility and concluded that this characteristics may not be very influent in biofilm formation.

Table 6-Motility of *A. calcoaceticus* and *S. maltophilia* with different concentrations of NaOCl. Values of motility halos diameter (mm) at different times (24, 48 and 72 h).

	A. calcoaceticus								
[NaoCl] (ppm)	**0**			**0.1**			**300**		
Time (h)	**24**	**48**	**72**	**24**	**48**	**72**	**24**	**48**	**72**
Swimming (mm)	0.0±0.0	10.0±2.8	52.0±3.0	1.0±0.0	18.0±6.9	45.0±3.5	2.7±0.5	4.5±0.7	10.0±4.0
Swarming (mm)	2.0±0.0	2.3 ± 0.6	2.3±0.6	1.5±0.7	2.3± 1.2	3.0 ± 1.0	3.3±1.5	4.0±1.0	4.2±1.2
Twitching (mm)	3.0±0.0	6.5 ± 0.7	6.7±0.6	2.0±0.0	2.7± 0.6	3.0 ± 1.0	2.0±0.0	3.0±1.0	3.3±0.6
	S. maltophilia								
[NaoCl] (ppm)	**0**			0.1			400		
Time (h)	**24**	**48**	**72**	**24**	**48**	**72**	**24**	**48**	**72**
Swimming (mm)	3.0±1.0	10.3±2.3	20.6±1.5	2.3±0.6	8.0±1.0	16.6±1.5	4.6±1.5	12.3±0.6	20.3±2.9
Swarming (mm)	2.7±1.5	5.6 ±1.2	7.0±2.0	3.3±1.5	5.3± 1.5	7.3 ± 2.4	2.0±1.4	3.5±0.7	5.0±1.4
Twitching (mm)	2.3±1.2	4.0 ± 0.0	4.3±1.0	2.3±0.6	3.3± 0.6	4.3 ± 0.6	3.3±0.6	4.3±0.6	5.0±1.0

3.3.4. Coaggregation

To understand how *A. calcoaceticus* and *S. maltophilia* can interact, their coaggregation and autoaggregation abilities were assessed. Autoaggregation is the ability of bacteria to recognize and adhere to genetically similar bacteria (Rickard et al. 2003b). On

the other hand, coaggregation is the highly specific recognition and adhesion of genetically distinct bacteria. This recognition specificity is mediated by some proteins present in cell surface, such as adhesion proteins and polysaccharide receptors (Kolenbrander 2000; Rickard et al. 2003a). Coaggregation may also influence biofilm development through two different ways: (1) it allows single cells in suspension to specifically recognize and adhere to genetically distinct cells in the biofilm and (2) cells previously coaggregated in suspension can also be secondary colonizers adhering to the developing biofilm (Rickard et al. 2003a).

A. calcoaceticus can form autoaggregates due to recognition of genetically similar cells (score 2/3 after 48h - Table 7) and this values are in accordance with those previously described by Simões et al. (2008a).

Table 7-Auto and coaggregation scores obtained over time for *A .calcoaceticus* and *S. maltophilia* exposed at different concentrations of NaOCl (score: 0- no visible coaggregates in the cell suspension; 1- very small uniform coaggregates in a turbid suspension; 2- easily visible small coaggregates in a turbid suspension; 3- clearly visible coaggregates which settle; 4 - very large flocs of coaggregates that settle almost instantaneously).

[NaOCl] (ppm)	**0**				**0.1**				**MIC**			
Time (h)	**0**	**2**	**24**	**48**	**0**	**2**	**24**	**48**	**0**	**2**	**24**	**48**
A.calcoaceticus/A. calcoaceticus	2	2	2	2/3	2	2	2	2/3	0	0	0	0
S. maltophilia/S. maltophilia	1	1	1/2	1/2	0	1	1/2	1/2	0	0	0	0
A.calcoaceticus/S. maltophilia	1/2	2	2	2	1/2	2	2	2	0	0	0	0

S. maltophilia has lower ability to autoaggregate (score 1/2 after 48 h) when compared to *A. calcoaceticus* (score 2/3 after 48 h), forming smaller aggregates over time. This is probably due to its lower ability to recognize similar cells. However, Rickard et al. (2003b) demonstrated that *S. maltophilia* B9 strain was not able to autoaggregate.

The effects of NaOCl on auto and coaggregation was also tested. The residual concentration of chlorine (0.1 ppm) appears not to have effects on autoaggregation of both bacteria, except for *S. maltophilia* in the first time, where aggregates were not visible (score 0). NaOCl at MIC caused the inhibition of all cell recognition and aggregation (Score 0).

The ability of *A. calcoaceticus* and *S. maltophilia* to coaggregate appears not be relevant, since the aggregates formed can be due to bacterium autoaggregation. To

conclude about the coaggregation ability it is necessary to perform additional tests, like microscopic visualization of aggregates and study the behavior of cell receptors responsible for the coaggregation process (adhesins and polysaccharides) by heat and protease treatment and sugar reversal tests (Simões et al. 2008a).

3.3.5. Bacterial adhesion to PVC

3.3.5.1. Prediction of adhesion

In order to predict the ability of the tested bacteria to adhere to PVC surfaces, the free energy of interaction between the two surfaces, when immersed in water, was calculated according a thermodynamic approach. The effects of NaOCl on the adhesion process were also assessed (Table 8). Using this approach, it is assumed that two particles immersed in an aqueous solution can approach to each other through different interactions: electrodynamics or Lifshitz-van der Waals interactions, polar or Lewis interactions, electrostatic interaction and interactions resulting from Brownian movements (Chaves 2004).

Table 8- Free energy of adhesion (ΔG_{1w2}^{TOT}) of *A. calcoaceticus* and *S. maltophilia* to PVC subjected to different NaOCl concentrations.

	ΔG_{1w2}^{TOT} (mJ/m^2)		
[NaOCl] (ppm)	**0**	**0.1**	**MIC**
A. calcoaceticus	1.1 ± 0.4	-2.9 ± 1.0	6.2 ± 0.7
S. maltophilia	6.6 ± 0.9	-5.1 ± 1.0	-1.5 ± 1.2

A. calcoaceticus and *S. maltophilia* showed positive values of free energy of adhesion: 1.1 ±0.4 and 6.6 ± 0.9 mJ/m^2, respectively. When these values are positive the adhesion is considered not be thermodynamically favorable (Simões et al. 2007a). When comparing the present results with previous studies, Simões et al. (2007a) found that *A. calcoaceticus* adhesion to PVC was thermodynamically feasible, since the achieved value of ΔG_{1w2}^{TOT} was -12.2 mJ/m^2. Those authors also found that only one of the two strains of *S. maltophilia* studied was thermodynamically unable to adhere to PVC surface (ΔG_{1w2}^{TOT}= 3.9 mJ/m^2). As referred previously, the different growth medium used affects the bacterial

surface properties, therefore it can explain the differences between the obtained results and those present in literature.

The addition of NaOCl at 0.1 ppm to the cell suspensions caused a significant decrease in the free energy of interaction between the two bacteria and PVC ($p<0.05$), causing thermodynamically favorable adhesion ($\Delta G_{1w2}^{TOT}<0$). However, the exposure of *A. calcoaceticus* to NaOCl at MIC caused an increase of free energy of adhesion to PVC becoming adhesion thermodynamically unfavorable. The opposite happened with *S. maltophilia* that becomes thermodynamically able to adhere to the surface due to the exposure to NaOCl at MIC ($\Delta G_{1w2}^{TOT} = -1.5 \pm 1.2$ mJ/m^2).

3.3.5.2. Experimental adhesion to PVC

The ability of cells to adhere to PVC coupons and the effects of NaOCl on the adhered cells was tested with the selected bacteria as single and co-cultures. Although ΔG_{1w2}^{TOT} of *A. calcoaceticus* and *S. maltophilia* assumed a positive value, showing that the adhesion of these bacteria to PVC was theoretically unfavorable, the experimental results demonstrated ability to adhere. *A. calcoaceticus* shows less ability to adhere to PVC than *S. maltophilia*, in both single and dual cultures ($p<0.05$), contrary to the free energy of adhesion results. The differences between the adhesion ability based on thermodynamics and the experimental values obtained, it can be justified by the non consideration of microbiological aspects in the prevision of adhesion. Attending to the DLVO theory, electrostatic interactions are more repulsive to higher effective radius of particles. Therefore, bacteria can reduce its effective radius of interaction through some structures that facilitate the adhesion, such as flagella, extracellular polymers, fimbriae, pili and prosthecae (Chaves 2004).

When the bacterial consortium was incubated with PVC coupons, the adhesion was mainly influenced by the presence of *S. maltophilia*, being the number of *A. calcoaceticus* lower than that observed for the single culture ($p<0.05$) (Figure 4).

Residual concentrations of NaOCl (0.1 ppm) does not cause significant differences in cell adhesion to both single and co-cultures ($p>0.05$). Therefore, the residual disinfectant concentration usually maintained inside the DWDS is not able to remove recently adhered cells to pipe walls. However, the increase of biocide concentration to the MIC decreased

the numbers of adhered cells for all the cases studied. However, even when a higher concentration of NaOCl was applied *S. maltophilia* shown be the most resistant bacteria. The application of NaOCl at the MIC was responsible for the decrease of adhered *S. maltophilia* by 0.69 log and *A. calcoaceticus* by 0.89 log for the single species cultures. For the co-culture, the decrease of adhered cells was lower. For this situation, *A. calcoaceticus* becomes more resistant (reduction of 0.20 log) than *S. maltophilia* (reduction of 0.42 log). These results corroborates the findings of Simões et al. (2009; 2011a) that species association increases the resistance of biofilm bacteria to chemical treatments, even if in the early phases of biofilm formation.

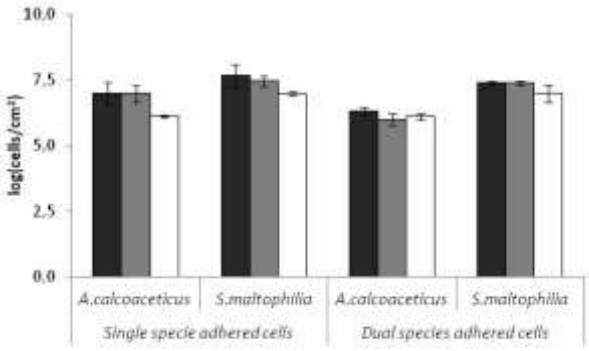

Figure 4- Effects of NaOCl on *A. calcoaceticus*, *S. maltophilia* and their consortium when adhered to PVC coupons for 2 h. ■ 0 ppm ■ 0.1 ppm □ MIC

3.3.6. The effects of NaOCl on OMP expression of A. calcoaceticus and S. maltophilia

Gram negative bacteria have an outer membrane which performs the crucial role of providing an extra layer of protection without compromising the exchange of required material for sustaining cell life (Delcour 2009).

The OMP profiles of *A. calcoaceticus* and *S. maltophilia* were assessed after treatment with different concentrations of NaOCl (0.1 ppm and MIC) in order to assess the effect of this chemical agent on OMP expression. Seven major proteins were identified in outer membrane of *A. calcoaceticus* with molecular weight between 25 kDa and 119 kDa (being all the weights: 25, 33, 40, 45, 49, 114 and 119 kDa). *S. maltophili*a major OMPs had molecular weight between 17 kDa and 131 kDa (being all the weights: 17, 21, 38, 47, 58, 62, 107, 118 and 131 kDa) (Figure 5).

A literature search revealed a 17 kDa subunit fimbriae (SMF-1) of *S. maltophilia* responsible for adherence and participation at early states of biofilm formation (De Oliveira-Garcia et al. (2003). Therefore, one of the OMPs detected in this work can correspond to SMF-1. The exposure of *A. calcoaceticus* and *S. maltophilia* to NaOCl at 0.1 ppm did not caused differences in the OMP expression. However, at the MIC no OMPs were found in the SDS-page gel. This is probably due to a strong interaction of NaOCl at the MIC and OMPs and/or limitations of the method. In fact, Virto et al. (2005) found significant damage of bacterial membranes when bacteria were exposed to chlorine at concentrations higher than 50 ppm.

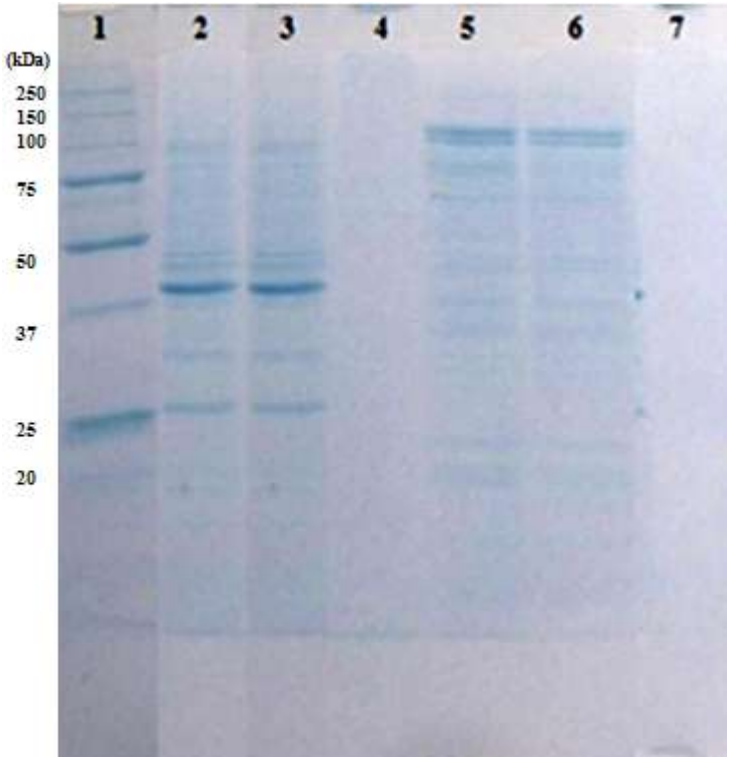

Figure 5- SDS-Page gel stained with Comassie Blue of OMP from *A. calcoaceticus* and *S. maltophilia*. 1- Protein marker; 2- *A. calcoaceticus*; 3- *A. calcoaceticus* treated with NaOCl at 0.1 ppm; 4- *A. calcoaceticus* treated with NaOCl at 300 ppm; 5- *S. maltophilia*; 6- *S. maltophilia* treated with NaOCl at 0.1 ppm; 7- *S. maltophilia* treated with NaOCl at 400 ppm.

3.4. Conclusions

The exposure to NaOCl changes some characteristics of *A. calcoaceticus* and *S. maltophilia*. The results showed that NaOCl becomes the surface of both bacteria more positive. However planktonic and adhered *A. calcoaceticus* are more affected by NaOCl

exposure than *S. maltophilia*, since a lower MIC was obtained to *A. calcoaceticus* as well as a more pronounced decrease on motility ability and changes in it hydrophobicity. Residual concentrations of NaOCl has not effect in OMPs expression and in autoaggregation ability of *A. calcoaceticus* and *S. maltophilia*. However NaOCl at the MIC inhibit these two cellular processes in both bacteria.

The adhesion was underestimated when predicted by the thermodynamic theory, being both bacterial effectively able to adhere to PVC. *S. maltophilia* had higher ability to adhere and was also more resistant to chemical removal than *A. calcoaceticus.* The association of both bacteria increased their resistance to removal, being this more significant for *A. calcoaceticus*. Any of the single and dual adhered bacteria was not susceptible to residual concentrations of NaOCl. The use of NaOCl at the MIC was able to decrease the numbers of adhered bacteria but complete removal from the surface was not achieved.

Chapter 4

4. Control of *A. calcoaceticus* and *S. maltophilia* single and dual species biofilms with chemical and mechanical stresses

4.1.Introduction

DW biofilms are relatively thin, composed by patchy and non-uniformly distributed aggregates (Abe et al. 2012), since hydrodynamics conditions affect both biofilm development and detachment. DWDS are usually exposed to variable hydraulic conditions, ranging from non-flow to laminar and/or turbulent flow regimes. Stagnation causes the biofilm accumulation in DWDS (Ayoub and Malaeb 2006) while sudden flow variations causes biomass detachment and resuspension (Lehtola et al. 2006).

As referred in Section 2.3. several strategies are used to control biofilms in DWDS like the disinfectant addition and pipe flushing out. These strategies can have important influence in biofilm detachment. However, biofilm release from pipe walls is not always desired because it can cause the microbial resuspension, being this phenomenon a potential cause of health problems to the water consumers due to the spread of biofilm-entrapped pathogens (LeChevallier 1999).

Biofilm detachment is caused by a combination of different processes like erosion, abrasion and sloughing (Horn et al. 2003), occurring when external forces are higher than the internal strength of the matrix that holds the biofilm together. Two main mechanisms that can lead to biofilm detachment are the increase of external shear forces (varying the hydrodynamics conditions) and the decrease of internal strength (hydrolyzing polymeric biofilm matrix) (Horn et al. 2003). It is clear that changes in external strength like shear stress variations have an important role in biofilm structure and density (Abe et al. 2012). Liu and Tay (2001) found that higher detachment forces can form a more compact, stable and denser biofilm, having significant influence on the structure, mass transfer, production of EPS, metabolic and genetic properties of the biofilm. A similar conclusion was achieved

by Paris et al. (2007) who found that biofilms formed under continuous turbulent flow had a denser structure, are thinner and more resistant than when formed under laminar flow. Also, the biofilm porosity is affected by shear stress intensity. Higher shear stress leads to a less porous structure since it stimulates the EPS production increasing biofilm cohesion and decreasing the detachment rates (Zhang and Bishop 1994). Therefore, biofilm structure is not homogeneous, the density decreases and the porosity increases from the bottom to the top (Zhang and Bishop 1994; Derlon et al. 2008).

The use of disinfectants in DWDS can affect the internal strength of biofilms. Biocides and disinfectants have been the main weapon to control unwanted biofilms, acting either by stopping growth or allowing natural detachment from surface (Simões et al. 2003). Oxidizing disinfectants, such as NaOCl, may depolymerize EPS enabling the detachment of biofilms from surfaces (Kumar and Anand 1998).

Some works were done in order to understand how can chemical treatments influence the biofilm behavior at varying hydrodynamic conditions (Simões et al. 2003, 2005; Simões et al. 2009; Brindle et al. 2011), however, this is almost unknown data for DWDS. Therefore, the aim of this study is to evaluate the effects of NaOCl in single and dual species biofilms formed by *Acinetobacter calcoaceticus* and *Stenotrophomonas maltophilia* and understand how can it influence the biofilm stability under varying hydrodynamics conditions.

4.2.Materials and methods

4.2.1. Microorganims and culture conditions

The microorganisms and culture conditions used for biofilm development are described in Section 3.2.1.

4.2.2.Biofilm formation in PVC coupons in microtiter plates

Biofilms were formed with *S. maltophilia*, *A. calcoaceticus* and their mixture using PVC as substratum. Cell suspensions were prepared as described in Section 3.2.2. The PVC coupons preparation and the microtiter plates inoculation was performed as described in Section 3.2.6.

The biofilm formation on PVC coupons was allowed to occur for 24 h at 23 ± 3ºC in an orbital incubator (New Brunswick Scientific, I26, USA) at 120 rpm. The negative controls were obtained placing the PVC coupons in PBS without bacterial cells. The experiment was performed in triplicate and at the end of the assay each coupon was washed with PBS, removing all the biofilm formed and resuspending it in 1 mL of PBS. The biofilm suspensions were filtered (0.5 mL) using a 0.22 µm black polycarbonate membrane (Nuclepore). The membrane with biofilm bacteria was observed using an epifluorescence microscope Leica DM LB2 (Leica Microsystems, Germany) after staining with 400 µL of DAPI (Sigma) at 0.5 µg/mL as described in Section 3.2.6.

The same experiment was performed testing the effects of NaOCl at 0.1 ppm and at the MIC (400 ppm to *S. maltophilia* and to the consortium, 300 ppm to *A. calcoaceticus*). After 24 h of incubation, the PVC coupons were exposed for 30 minutes to the biocide (1.2 mL) at the referred concentrations.

4.2.3. Rotary cylinder reactor - experimental set-up

Biofilms were grown on PVC cylinders with a surface area of 34.6 cm^2 (diameter = 2.2 cm; length = 5.0 cm) inserted in a 5 L reactor, maintaining a shear stress of 0.09 Pa. Three PVC cylinders were used in every experiments and the reactor was inoculated with 250 mL of cell suspension (*S. maltophila* and *A. calcoaceticus*), grown as described in Section 3.2.1., before starting the operation, being continuously fed with sterile diluted medium (0.05 g/L glucose, 0.025 g/L peptone, 0.0125 g/L yeast extract and 0.2 M phosphate buffer at pH7) at a constant rate (0.5 $L.h^{-1}$). The biofilms were allowed to grow for seven days in order to obtain steady-state biofilms (Pereira et al. 2002). In each day it was added 1 mL of cell suspension in order to avoid any possible event of complete bacterial wash-out.

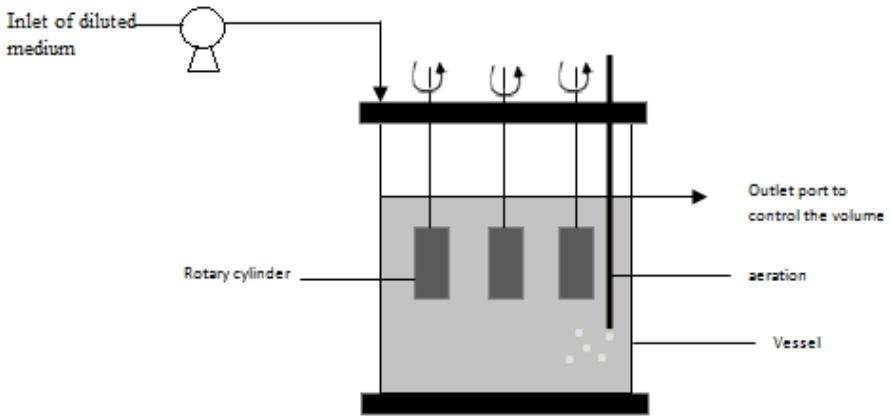

Figure 6- Sectional view of the rotary cylinder reactor.

4.2.3.1.Mechanical stability

The mechanical stability of the biofilms was assessed by means of determining the biomass loss weight due to the exposure of biofilms to increasing the shear stress (0.22, 0.72, 1.42 and 2.31 Pa) in a rotary cylinder device as described Azeredo and Oliveira (2000). To determine the shear stress it was used the Equation 4 and it is controlled by adjusting the rotation speed.

$$\tau_s = \frac{f.\rho.v^2}{2} \qquad \text{(Eq.4)}$$

where τ_s is the shear stress, f is the fanning factor and v is average velocity (m/s) (Altman et al. 2009).

The fanning factor to a rotary cylinder at Reynolds number of agitation higher than 300 is given by the Equation 5 as described Gabe and Walsh (1983).

$$f = 0.158 Re_A^{-0.3} \qquad \text{(Eq.5)}$$

$$Re_A = \frac{D^2 N \rho}{\mu} \qquad \text{(Eq.6)}$$

where Re_A is the Reynolds number of agitation, D (m) is the diameter of the cylinder, N (s^{-1}) is the rotation speed, ρ (Kg/m^3) is the fluid density and μ (Kg/m s) is the fluid viscosity (Geankoplis 1993).

After seven days of formation, the cylinders *plus* biofilm were carefully removed from above referred 5 L reactor. One of the cylinders was then immersed in a reactor with

0.2 M phosphate buffer (the control cylinder). While the others were immersed in 300 mL reactors containing NaOCl solutions at 0.1 ppm and MIC. This chemical treatment was carried out with cylinders under constant shear stress (0.09 Pa) during 30 minutes. Afterwards, the cylinders were removed from the reactors containing the chemical solutions, accurately weighed, introduced in other reactors with phosphate buffer and consequently subjected to serial shear stress, i.e. 0.22 Pa, 0.72 Pa, 1.42 Pa and 2.31 Pa for a period of 30 seconds each.

The wet weight of the cylinder *plus* biofilm attached was determined before and after each exposure to a different shear stress. The experiments were repeated in three different occasions for every chemical treatment tested.

The wet mass of the biofilm that was removed from the surface area of each cylinder, after each shear stress exposure, was expressed in percentage of biofilm removal (Equation 7) and the amount of biofilm that remained adhered after submission to the complete series of shear stress was expressed as percentage of biofilm remaining according Equation 8 (Simões et al. 2005).

$$\%\ biofilm\ removal_i = \frac{X_{after\ NaOCl} - X_i}{X_{after\ NaOCl} - X_c} \times 100 \qquad \text{(Eq.7)}$$

where $X_{after\ NaOCl}$ is the cylinder weight after being exposed to NaOCl during 30 minutes, X_i is the cylinder weight after being subjected to the i shear stress and X_c is the wet mass of PVC cylinder without biofilm.

$$\%\ biofilm\ remaining = \frac{X_{2.31Pa} - X_C}{X_{after\ NaOCl} - X_C} \times 100 \qquad \text{(Eq.8)}$$

where $X_{2.31Pa}$ is the cylinder weight after being exposed during 30 seconds to a shear stress of 2.31 Pa.

4.2.4.. Statistical analysis

The statistical analysis was performed as referred in Section 3.2.12.

4.3.Results and discussion

4.3.1. Effect of NaOCl in recently formed biofilms

Microtiters plates with PVC coupons were used as reactor to form single and dual species 24 h-aged biofilms. The effect of NaOCl to remove biofilms assessed through microscopic analysis, allowing to evaluate reductions in cell density of biofilms formed on PVC coupons. Figure 7 presents the behavior of each biofilm formed after exposure to NaOCl at 0.1 ppm and the MIC for 30 minutes.

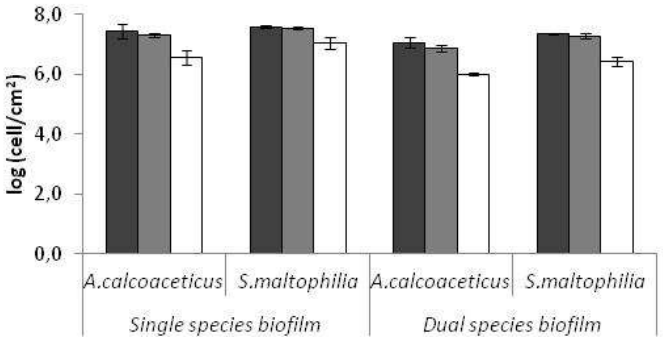

Figure 7- Effects of NaOCl in the removal of 24 h- aged biofilms formed on PVC coupons. *A. calcoaceticus* - MIC is 300 ppm; *S. maltophilia* and dual species biofilm - MIC is 400 ppm.

Residual concentrations of chlorine have not a significant effect on the removal of attached cells from the 24 h-aged single and dual species biofilms ($p>0.05$). However, the exposure to NaOCl at the MIC caused a significant reduction of the biofilm cells ($p<0.05$). This reduction was 0.88 log for *A. calcoaceticus* and 0.54 log for *S. maltophilia* in single species biofilms. This result shows that the last bacteria is more resistant to chlorine action than *A. calcoaceticus* ($p<0.05$).

The reduction of attached cells in dual species biofilm at the MIC values was also significant ($p<0.05$) but there are no evidences that these biofilms are more robust and resistant than those single species. *A. calcoaceticus* reduction in the dual species biofilms was 1.05 log while *S. maltophilia* reduction was 0.92 log. Comparing these results with those of bacterial adhesion to PVC coupons (Section 3.3.5.), it is possible to observe that

single species biofilms and the adhered cells had similar behavior to NaOCl treatment, being *A. calcoaceticus* more susceptible to NaOCl than *S. maltophilia*. However, the dual species adhered cells do not predict the biofilm behavior. It was observed a higher resistance of dual species adhered cells to chemical treatments contrary to the obtained to the correspondent biofilm. These results can explain that the initial layer of cells that starts building the dual biofilm is apparently more resilient to chemical treatment than the further layers. Therefore, when the biofilm is formed and developed, the superficial layer can be easily affected by NaOCl being possible to observe a higher bacterial removal rates in biofilms than in adhered cells. Although NaOCl is not able to remove completely the biofilm from PVC, additional tests (rezasurin method) were done in order to assess the ability of NaOCl to inactivate the bacteria attached inside the biofilm matrix. It was achieved that this disinfectant is also able to inactivate bacteria within biofilms, being also *A. calcoaceticus* the most susceptible. However the application of NaOCl even at MIC do not allow to achieve the complete bacteria inactivation (data not shown).

4.3.2. Chemical and mechanical treatment of steady-state single and dual species biofilms

It was used a rotary cylinder reactor where was formed biofilm during 7 days to achieve a steady state in its phenotype (see Figure B.1 in the Appendix B). The biofilms formed on the surfaces of the PVC cylinders were exposed to chemical treatment during 30 minutes being then subjected to a mechanical treatment, changing the hydrodynamics conditions at each 30 seconds. It was evaluated the effect of exposure to different shear stress (0.22, 0.72, 1.42 and 2.31 Pa) in order to simulate the biofilm behavior in DWDS after flushing out the pipes. All these shear stresses are included in the commonly range existent in channels within biofilms (Bakker et al. 2003). Douterelo et al. (2013) also used similar shear stress (0.2 to 3 Pa) to simulate a network flushing operation, the application of 0.2 Pa corresponds to a situation before flushing and exposure at 3 Pa mimics a flushing scenario. These procedures allow to determine the influence of chemical treatment in biofilms hydrodynamic stability.

4.3.2.1.Effects of NaOCl treatment

The biofilms formed on PVC cylinders surfaces were exposed to different concentrations of NaOCl (0.1 ppm and MIC). This disinfectant is an oxidizing agent able to

inhibit the metabolic activity since it oxidize membrane and cytoplasmatic enzymes with thiol groups (Denyer and Stewart 1998). However, the biocide efficiency is affected by the presence of biofilms. Tachikawa et al. (2005) compared the efficiency of different biocides and found that NaOCl is the most affected by presence of biofilms, reducing its disinfectant potential due to reaction with EPS.

Figure 8 presents the percentage of biofilm removal from PVC surface after treatment with NaOCl at 0.1 ppm and at MIC. Attending to the *A. calcoaceticus* biofilm behavior it is possible to observe a removal of 23.5 ± 7.3% from the PVC cylinder after being exposed during 30 minutes to a solution of NaOCl at 0.1 ppm. A similar result was obtained, at same conditions, to the single species biofilms formed by *S. maltophilia* (removal of 13.6 ± 3.4%) ($p>0.05$). At this concentration, the dual species biofilm is more susceptible to chemical removal as was achieved to 24 h-aged biofilms (Section 4.3.1.), being removed 45.7 ± 6.9% of the formed biofilm (Figure 8).

The exposure to NaOCl at the MIC caused similar removals of single and dual species biofilms ($p>0.05$). However, comparing the application of different NaOCl concentrations, it is possible to observe that the use of NaOCl at the MIC increases significantly the single species biofilm removal compared to the dual biofilms ($p<0.05$). In fact, its effect in dual species biofilms is similar to that caused by the treatment with 0.1 ppm ($p>0.05$).

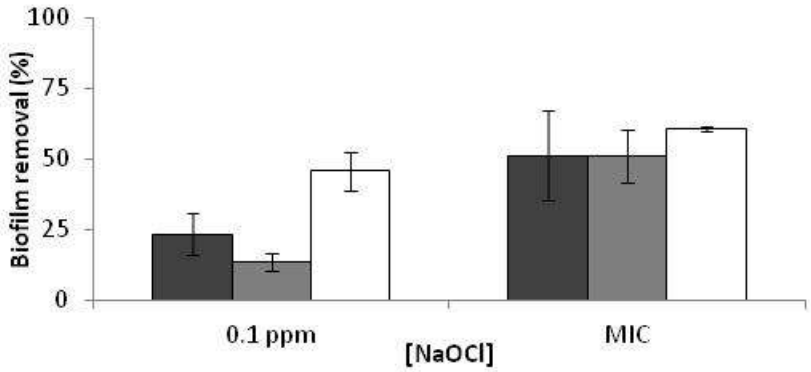

Figure 8- Removal of *A. calcoaceticus* and *S. maltophilia* single and dual species biofilms induced by the exposure to different concentrations of NaOCl for 30 minutes.

4.3.2.2.Effect of mechanical stress after the exposure to NaOCl treatment

Figure 9 shows the single and dual species biofilms removal caused by the implementation of a series of increasing shear stresses after the biofilms being treated with NaOCl at different concentrations.

Attending to the behavior of untreated *A. calcoaceticus* biofilm (Figure 9.A) when exposed to different mechanical stresses it is possible to observe the highest removal percentages with shear stresses of 0.72 (20.8 ± 0.4%) and 1.42 Pa (19.1 ±7.7%) ($p>0.05$). The exposure to 0.22 and 2.31 Pa caused similar biofilm removal ($p>0.05$), with values of 10.5 ± 2.6% and 9.5 ±1.4%, respectively. Therefore, the intermediate shear stresses demonstrated to have more influence on *A. calcoaceticus* biofilm removal.

NaOCl changes the mechanical stability of *A. calcoaceticus* biofilms. The pretreatment of *A. calcoaceticus* biofilm with 0.1 ppm of NaOCl caused higher removal at 0.22 and 2.31 Pa, being removed 21.4 ± 3.0% and 16.7 ± 9.3% of the total biofilm mass, respectively. At 0.72 Pa, 10.5 ± 3.5% of the biofilm mass was removed. The increase of the shear stress to 1.42 Pa did not caused significant biofilm removal. So, the pretreatment with 0.1 ppm NaOCl makes the biofilm physically more stable at intermediate shear stress (0.72 and 1.42 Pa). The pretreatment with NaOCl at MIC caused an increase of *A. calcoaceticus* biofilms mechanical removal with the application of higher shear stress. The exception was with 2.31 Pa that caused a residual removal (1.9 ± 0.6%). This result proposed that NaOCl at MIC induces higher resistance to 2.31 Pa exposure.

Figure 9.B presents the *S. maltophilia* single biofilms behavior to different shear stresses. When *S. maltophilia* biofilm was not subjected to the chemical treatment the higher removal was achieved with the lower shear stress applied (0.22 Pa), being removed 29.9 ± 4.0% of the total biofilm. The application of shear stresses of 0.72 and 2.31 Pa had similar effect on the biofilm mechanical stability ($p>0.05$), being removed 17.1 ± 0.5% and 17.5 ± 0.4% of its biofilm mass, respectively. Pretreatment with NaOCl at 0.1 ppm becomes the biofilm more resistant to all the shear stresses applied, as happened with the treatment with NaOCl at MIC that increased biofilm resistance to the shear stress. In fact, biofilm removal (14.0 ± 0.4%) was only found at 0.72 Pa. Comparing the effects of NaOCl

concentration on *S. maltophilia* biofilm stability, it is possible to observe an increase of mechanical stability when pre-exposed to NaOCl at high concentrations.

The presented results show that *A. calcoaceticus* biofilms were more stable to shear stresses of 0.22 and 2.31 Pa than those of *S. maltophilia*. The chemical pretreatment at 0.1 ppm and the MIC becomes *S. maltophilia* biofilms more stable to hydrodynamics changes than *A. calcoaceticus* biofilms ($p<0.05$).

Figure 9.C shows data on the evaluation of the mechanical behavior of dual species biofilms after exposure to NaOCl. Chemically untreated biofilms were susceptible to intermediate shear stress (0.72 and 1.42 Pa) and more resistant to the application of higher shear stress (2.31 Pa), having a similar behavior in biomass removal to the *A. calcoaceticus* biofilms, particularly for shear stresses of 0.72, 1.42 and 2.31 Pa ($p>0.05$).

When dual species biofilms were previously treated with NaOCl at 0.1 ppm, biofilm removal was only found for intermediate shear stresses, 0.72 and 1.42 Pa (17.0 ± 6.9% and 10.0 ± 4.4 %). The use of NaOCl at MIC caused highest removal (47.9 ±1.8%) at low shear stresses (0.22 Pa). It is visible a decrease of biofilm removal percentage (from 20.3 ± 4.7 % to 11.7 ± 4.2 %) with the increase of shear stress (from 0.72 to 1.42 Pa). No biofilm removal was found with the implementation of a shear stress of 2.31 Pa. Comparing the influence of NaOCl at 0.1 ppm and MIC on mechanical stability of biofilm, it was found that the first one becomes the biofilm more resistant to mechanical stress.

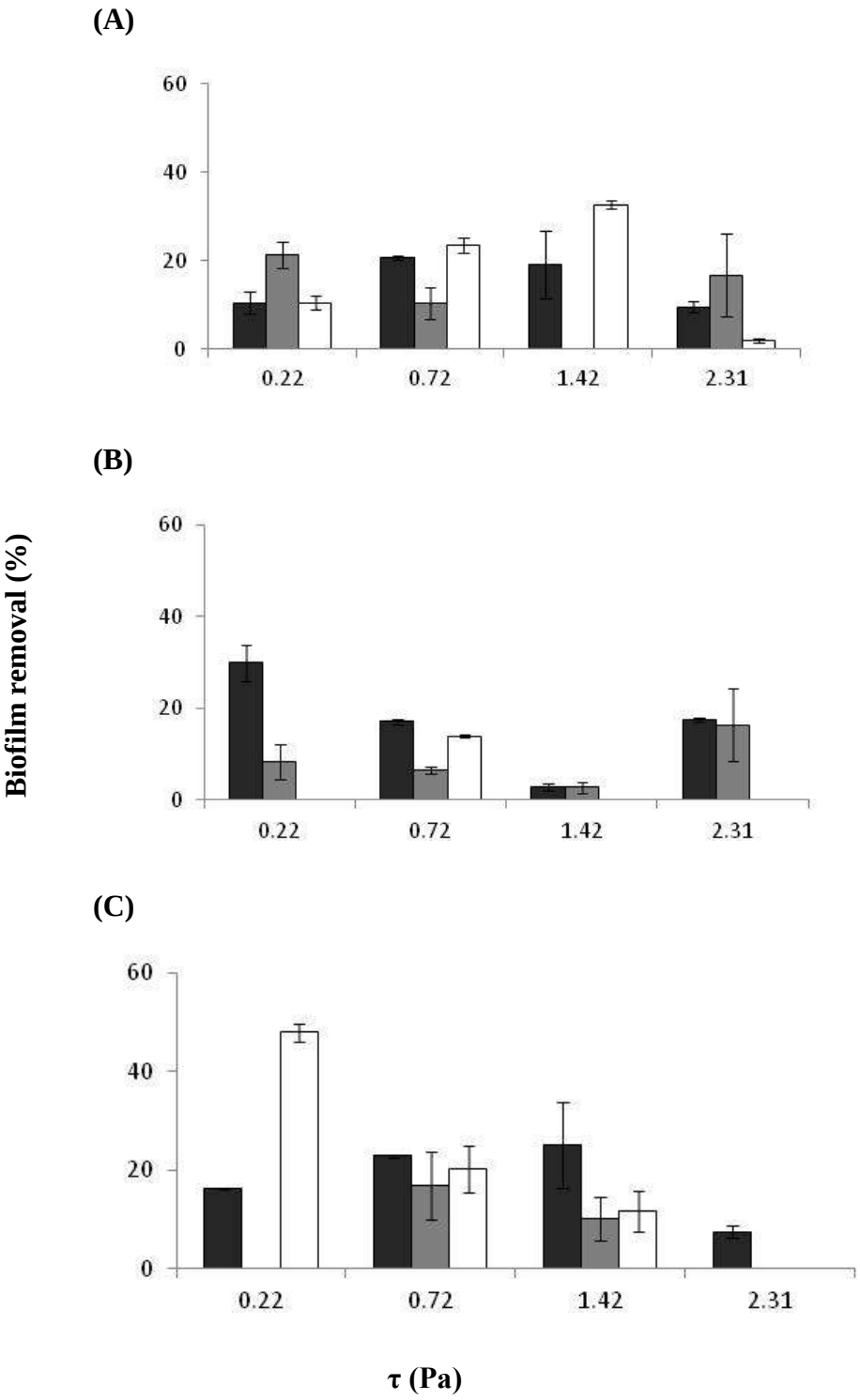

Figure 9- Biofilm removal from PVC cylinders due to exposure to a series of increasing shear stress conditions. A- *A. calcoaceticus* single species biofilms; B- *S. maltophilia* single species biofilms; C- Dual species biofilms.

4.3.2.3.Biofilm remaining after chemical and mechanical treatments

NaOCl reacts strongly with the EPS, depolymerazing it and destroying the biofilm structure that changes it hydrodynamic vulnerability (Kumar and Anand 1998; Simões et al. 2005). Therefore, after being treated with different concentrations of NaOCl and being exposed to an increasing series of shear stress, the biofilms were not completely removed from PVC surfaces. Figure 10 presents the percentage of biofilm that was not removed from the cylinder surface. These biofilms that remain on the PVC can have an important role in biofilm regrowth.

The previous chemical treatment with NaOCl at 0.1 ppm and at MIC had no significant effects on the efficiency of mechanical removal of *A. calcoaceticus* biofilms, being the percentage of remaining biofilm on PVC surface (Figure 10) similar in chemically treated and non-treated cylinders ($p>0.05$). Therefore NaOCl only promotes different biofilm removal for different shear stresses, being the overall removal similar (Figure 9.A).

The comparison of *S. maltophilia* biofilms behavior exposed to NaOCl at 0.1 ppm and at the MIC shows that the biofilm becomes more recalcitrant to mechanical stress after being exposed to NaOCl at higher concentrations ($p<0.05$). Although this concentration caused higher chemical removal of *S. maltophilia* biofilm than the exposure to residual concentrations of NaOCl (Figure 8). It means that the basal layer of the formed biofilm is apparently more cohesive and resistant to mechanical stress than those located close to the bulk as described by Derlon et al. (2008).

S. maltophilia and *A. calcoaceticus* biofilms appeared to have similar chemical removal when exposed to different NaOCl concentrations (Figure 8). However, this pretreatment with NaOCl at MIC induces *S. maltophilia* biofilms to resist in a higher extent to the mechanical stress variation than *A. calcoaceticus* biofilms, remaining on the PVC surface 85.8 ± 11.4% of total biofilm mass.

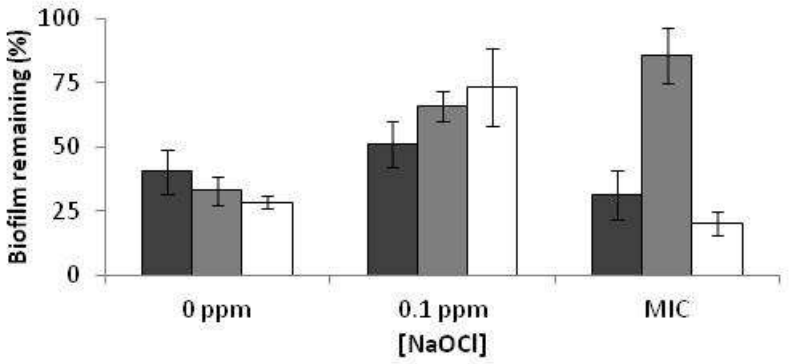

Figure 10- Biofilm remaining on the PVC surface after exposure to an increasing series of shear stress.

Despite the chemical removal of dual species biofilm was similar for both NaOCl concentration tested (Section 4.3.2.1), the exposure to NaOCl at 0.1 ppm induced a higher resistance to mechanical stress than the pretreatment with MIC ($p<0.05$). The biofilm remaining after treatment with 0.1 ppm was 73.4 ± 19.7% while with the MIC biofilm removal was 20.2 ± 4.9% (Figure 10). The different remaining percentages of biofilm and the different behavior face to shear stress variation (Figure 9.C) propose that higher concentrations of NaOCl weak the dual species biofilm structure.

4.4.Conclusions

S. maltophilia 24 h-aged biofilms are the most resistant to NaOCl removal. However, the presented results showed that NaOCl at 0.1 ppm is not able to remove 24 h-aged single and dual biofilms from PVC coupons, contrary to the higher NaOCl concentration that reduced the attached bacteria. The association of both bacteria did not confer additional resistance against NaOCl treatment.

The chemical removal of 7 d-aged biofilms caused by NaOCl at 0.1 ppm treatment was similar to *A. calcoaceticus* and *S. maltophilia* biofilms, being the dual species 7 d-aged biofilm less resistant to chemical removal than the single species biofilm. The use of NaOCl at MIC increased the single species biofilm removal, however the dual species biofilm removal caused by this treatment is similar to those caused by NaOCl at 0.1 ppm exposure. Comparing the behavior of 24 h-aged biofilms and 7 d-aged biofilms it was observed that the first ones are more resistant to chemical removal, mainly to 0.1 ppm.

The application of both concentrations of NaOCl caused changes in the 7 d-aged biofilms mechanical stability. The treatment with NaOCl at 0.1 ppm and at the MIC promoted different *A. calcoaceticus* biofilms removal for different shear stresses, however, the overall removal was similar to both concentrations. Contrary to *A. calcaoceticus* biofilms, NaOCl treatment makes *S. maltophilia* biofilm more resistant to mechanical stresses, increasing the resistance to removal for the higher NaOCl concentration tested. The pretreatment with NaOCl at 0.1 ppm becomes the dual species biofilm more resistant to mechanical stress than exposure to NaOCl at MIC, being the remaining biofilm after the shear stress series application higher in the first case referred.

This study clearly demonstrates the resistance of biofilms to both chemical and mechanical stresses, even if very high NaOCl concentrations and shear stress conditions were applied.

Chapter 5

5. Concluding remarks and research needs

5.1. General conclusions

Understanding the effect of NaOCl treatment in DWDS biofilms control, as well as it effects in biofilm mechanical stability may be important to improve the control of DWDS biofilms and reduce its impact in DW quality.

1) NaOCl altered some membrane properties: surface charge of both bacteria became more positive, the autoaggregation ability and the OMP expression of both bacteria only was affected by the exposure to NaOCl at MIC, being apparently inhibited.
2) *A. calcoaceticus* was the bacteria more susceptible to the treatment with NaOCl. *A. calcoaceticus* has a lower MIC and only its hydrophobicity and motility was affected by chemical treatment.
3) All the physicochemical properties influences the bacterial adhesion, however the adhesion was underestimated by the prevision done through thermodynamics approach. Both bacteria adhered to PVC but *A. calcoaceticus* was the bacteria with less ability to adhere to PVC surface, being also more affected by chemical treatment.
4) The use of NaOCl at residual concentrations has not effect in the removal of *A. calcoaceticus*, *S. maltophilia* and its co-culture from PVC surface in early stages of biofilm development (2 h adhered cells and 24 h-aged biofilms).
5) The biofilm behavior depends of its stage of development, being the resistance to chemical removal dependent of its age.

   (a) 2 h adhered cells: the more resistant was the co-culture, followed by the *S. maltophilia* and the less resistant was *A. calcoaceticus*

   (b) 24 h-aged biofilm: the more resistant was *S. maltophilia*, followed by *A. calcoaceticus* and then the dual species biofilm

(c) 7 d-aged biofilms: *S. maltophilia* and *A. calcoaceticus* biofilms have similar behavior face to chemical removal, however the dual species biofilm was the most susceptible to chemical removal.

6) The chemically untreated single and dual 7 d-aged biofilms have all similar behavior when exposed to the series of increasing shear stress.
7) The treatment with NaOCl changes the biofilm behavior when exposed to mechanical stress.
   (a) The exposure to different concentrations of NaOCl does not affect the efficiency of *A. calcoaceticus* mechanical removal, being the remaining biofilm after the exposure to the shear stresses series similar in all the cases.
   (b) *S. maltophilia* biofilm becomes more resistant to mechanical stress after being exposed to NaOCl. The pretreatment with NaOCl at the MIC induces *S. maltophilia* biofilms to resist in higher extent to the mechanical stress variation than *A. calcoaceticus* biofilms.
   (c) The exposure to NaOCl at MIC makes the dual species biofilm weaker against the hydrodynamic stress.

Attending to all the results it is possible conclude that residual concentration of NaOCl normally present in DWDS is not efficient in recently and steady-state biofilms control. Biofilms are resistant to both chemical and mechanical stresses, even if very high NaOCl concentrations and shear stress conditions were applied. Therefore, the combination of chemical treatment and pipe flushing is not able to remove it completely, so the remaining biofilm on PVC surface can influence cell attachment and biofilm regrowth in DWDS.

5.2. Future work

After the study of bacterial surface characterization some doubts remained about bacterial coaggregagtion ability, so it is necessary perform additional tests, like microscopic visualization of aggregates and study the behavior of cell receptors responsible by coaggregation process (adhesin and polysaccharides) by heat and protease treatment and sugar reversal tests.

To complete the study about the chemical and mechanical influence in biofilm removal, a phenotypic characterization of the biofilms formed in the reactor (such as cell

density and EPS quantification, as well as cell viability) should be done, allowing to understand the different characteristics of the different biofilms. The study should also include other biocide concentrations in order to determine if there is one concentration that enhance significantly the biofilm removal and if it is adequate to DWDS application.

Both bacteria are described in literature as potential human pathogens. Therefore it will also be interesting to study the antimicrobial resistance phenotype, being possible to determine if these bacteria are resistant to the action of some of the most used antibiotics, evaluating the risks of its presence in DWDS to human health.

It will be interesting to test the regrowth of the remaining biofilm and its behavior to new control conditions.

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Appendix

Appendix

Appendix A

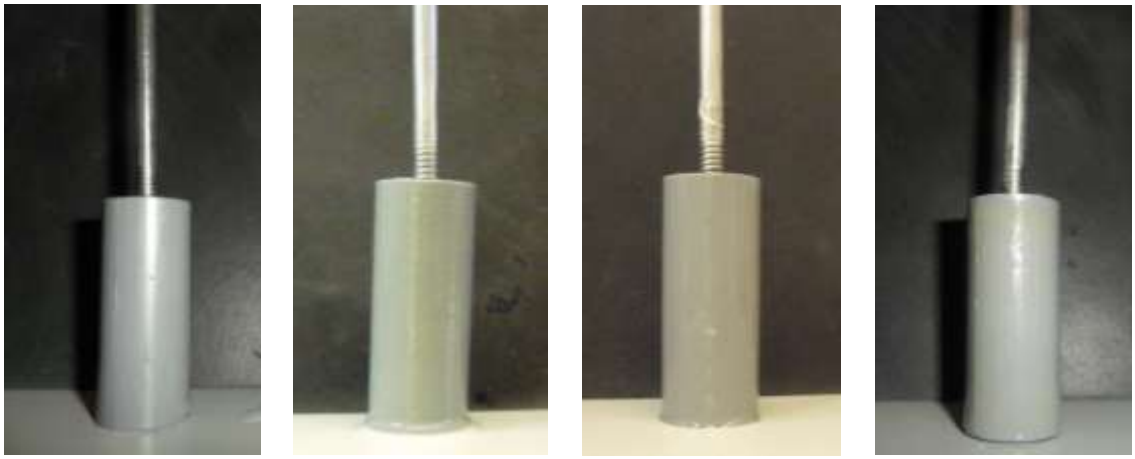
In this Appendix is presented Table A.1 with the values of PVC hydrophobicity parameters obtained from Simões et al. (2007a).

Table A.1- Surface tension parameters (γ^{LW}- Lifshitz- van der Waals component ; γi+- electron acceptor parameter ; γi-- electron donor parameter; γAB - Lewis acid-base component)and hydrophobicity (ΔG_{iwi}^{TOT}) characteristics of PVC (Simões et al. 2007a)

Material	**Surface tension parameters (mJ/cm^2)**			
	γ_{lw}	γ_i^+	γ_i^-	ΔG_{iwi}^{TOT}
PVC	33.9	0.0	5.8	-55.9

Appendix B

In this appendix are presented the figures of PVC cylinders used in the biofilm formation inside the rotating cylinder reactor.



Clean cylinder | *A. calcoaceticus* biofilm | *S. maltophilia* biofilm | Dual species biofilm

Figure B.1- PVC cylinders after 7 days of biofilm development in rotating cylinder reactor.