Master Degree in Bioengineering

Marine Biofilms – How do They Start?

Dissertation for Master Degree in Bioengineering

Specialization in Biological Engineering

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There is only one thing that makes a dream impossible to achieve:

the fear of failure

Paulo Coelho, *The Alchemist*

Acknowledgments

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Finally, a thank you God for all the strength you give me to carry on every day.

Resumo

Atualmente, os biofilmes estão presentes em todos os lugares do planeta e fazem parte do quotidiano do ser humano. Os biofilmes podem ser benéficos ou prejudiciais para a humanidade. Os biofilmes marinhos podem constituir um grande problema quando se formam em estruturas fabricadas pelo Homem como por exemplo os navios e plataformas de petróleo que se encontram em ambientes marinhos. De forma a descobrir estratégias “anti-fouling” que previnem o desenvolvimento de biofilmes marinhos sem prejudicar o ecossistema que os rodeia, é fundamental estudar os princípios da sua formação. Deste modo, o foco desta investigação foi o estudo da influência de tensões de corte e material da superfície no crescimento de biofilmes de *Pseudoalteromonas tunicata* (bactéria marinha).

Inicialmente, a curva de crescimento específica foi determinada e um valor igual a 0.533 h^{-1} foi obtido. A seguir, microplacas de 6 poços foram usadas de modo a avaliar o efeito de duas tensões de corte (0.1 e 1 Pa) na adesão da *P. tunicata* em aço inoxidável e cloreto de polivinilo (PVC).

O método de remoção do biofilmes envolvendo a agitação do cupão pelo vórtex provou ser ineficaz para a remoção de *P. tunicata* das duas superfícies testadas o que indicou que os biofilmes formados por esta bactéria possuem maior coesão em comparação com biofilmes formados, nas mesmas condições, por *Escherichia coli*.

Palavras-chave: Biofilmes marinhos, “Pseudoalteromonas tunicata”, adesão bacteriana, propriedades de superfície, tensão de corte

Abstract

Biofilms are present in every day of everyone's life and exist in every corner of the planet and can be beneficial or detrimental for humans. Marine biofilms can be very detrimental when formed in man-made structures such as ships and offshore oil platforms that are located in marine environments. In order to discover antifouling strategies that prevent the development of marine biofilms without harming the surrounding environment, it is fundamental to understand the principles behind their formation. Therefore, the focus of this work was the study of the influence of shear stress and surface material in the biofilm formation of *Pseudoalteromonas tunicata* (a marine bacterium).

In a first step the specific growth rate was determined and a value of 0.533 h^{-1} was obtained. In a second step, 6-well microtiter plates were used in order to evaluate the effect of two shear stresses (0.1 and 1 Pa) in the adhesion of *P. tunicata* to Stainless Steel (SS) and Polyvinyl chloride (PVC).

A biofilm detachment method involving coupon vortexing in saline solution proved to be ineffective for the removal of *P. tunicata* from both tested surfaces indicating that the biofilms formed by this bacteria have greater cohesion than those formed in similar conditions by *Escherichia coli*.

Keywords: Marine biofilms, "Pseudoalteromonas tunicata", bacterial adhesion, surface properties, shear stress

Declaração

Eu, Fábio Miguel Fernandes Figueiredo, declaro, sob compromisso de honra, que este trabalho é original e que todas as contribuições não originais foram devidamente referenciadas com identificação da fonte.

29 de Junho de 2015

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List of Abbreviations

ASW – Artificial Sea Water

BAC – Benzalkonium chloride

BDMDAC – Benzyldimethyldodecylammonium chloride

CFU – Colony Forming Units

DAPI – 4’,6-diamidino-2-phenylindole

EPS – Extracellular Polymeric Substance

MBC – Minimum Bactericidal Concentration

MIC – Minimum Inhibitory Concentration

NSW – Natural Sea Water

PVC – Polyvinyl Chloride

SS – Stainless Steel

QAC – Quaternary ammonium compound

VNSS – Vaatanen Nine Salt Solution

1. Introduction

1.1. Thesis Project Presentation and Main Objective

Most microorganisms live in aggregated communities designated as Biofilms (Flemming and Windenger, 2010). In the last years, many researchers were involved in exploration of biofilms because they are detrimental in many aspects of our lives (Callow and Callow, 2002). So strategies and methods to control and/or kill biofilms are necessary. Marine biofilms are one of the examples of unwanted biofilms that brings many problems and costs especially to the naval industry (Schultza *et al.*, 2011).

The main objective of this thesis was to study the growth of marine biofilms on different surfaces and different hydrodynamic conditions in order to understand the effect of these parameters on biofilm development. Additionally, the effect of a biocide on biofilm control was also evaluated.

1.2. Thesis Organization Structure

The contents of the thesis are divided into chapters, each one exploring different aspects and subjects.

In the first chapter, it is made an introduction to the focus of this thesis, summarizing the reasons that led to the development of this investigation and the description of the main objectives.

The second chapter constituted by theoretical fundaments, is a review of the background of marine biofilms. Factors that affect the formation of marine biofilms, methods to control biofouling, bacterial adhesion study methods are covered in this chapter.

In the Material and Methods chapter, the methods and protocols of the experiments made in the laboratory for this project are summarized. The results obtained by those experiments are presented and discussed in the fourth chapter, Results and Discussion. Chapter 5 (Conclusions and Future Outlook) refers to the main conclusions taken by the analysis of the results and suggestions for future work.

2. Theoretical Fundamentals

2.1. Biofilms

Microorganisms can be found in every place on Earth and usually they live as biofilms (Hilary *et al.*, 1989). Biofilms are organised communities of microorganism, surrounded by an extracellular polymeric substance (EPS) secreted by them, with an intricate architecture composed by channels which enable nutrient and metabolites transport through this structure. Bacteria are the most common microorganisms that compose a biofilm. The process of biofilm formation starts with planktonic bacterial adhesion to a surface by the use of extracellular sticky appendages (reversible adhesion). After an adaptation of the biofilm metabolic state, bacterial colonies start the production of EPS (irreversible adhesion). Living as biofilms enables bacteria to resist to hostile environments and antibiotics/biocides as well as improves their access to nutrients and confers a more resilient colonisation (Jakob *et al.*, 2008).

Usually biofilms are detrimental and appear in water containing environments (industrial pipe lines, heat exchangers, ship hulls, teeth, etc). Biofilms can cause heat transfer reduction, increase in fluid frictional resistance, energy loss, efficiency reduction, material deterioration, reduction in water quality by bacterial detachment and pathogens release, decrease in product quality, dental plaque and caries formation, etc (Hilary *et al.*, 1989). The main industries that suffer from those consequences are the food, chemical, power, shipping and metal industries. In addition, biofilms can also affect the human health (e.g. dental health) and municipal facilities, as drinking water pipelines (Characklis *et al.*, 1982; Lisbeth *et al.*, 2003).

2.2. Marine Biofilms

Marine biofilms are an example of undesired biofilms (Callow and Callow, 2002) which can be undesirable when they grow on man-made structures (ship hulls, power plant cooling systems, aquaculture systems, fishing nets, pipelines, submerged structures and ocean research instrumentation) causing several problems pipelines (Characklis *et al.*, 1982; Lisbeth *et al.*, 2003). In figure 1 it is possible to observe illustrative images of biofouling in ship hulls (Callow *et al.*, 2011).

Figure 1 – Shipp hulls with biofouling (adapted in Callow *et al.*, 2011)

When formed in ship hulls, biofilms lead to an increase in roughness which increases the hydrodynamic drag as the ship moves through water, enhancing fuel consumption (Callow and Callow, 2002; Inbakandana *et al.*, 2013). Biofouling may also lead to an increase in ship work stops for hull cleaning, may promote paint removal which leads to the need of repainting (having environmental consequences), which in turn, contributes to the increase of the total cost related with shipping industry (Characklis *et al.*, 1982; Inbakandana *et al.*, 2013). Additionally, when formed in marine sensors, biofilms can decrease their life time and increase the maintenance costs associated with the equipment downtimes that are necessary for system cleaning (Whelan and Regan, 2006). Figure 2 shows the factors which contribute for the increase in the operational and support costs in the shipping industry.

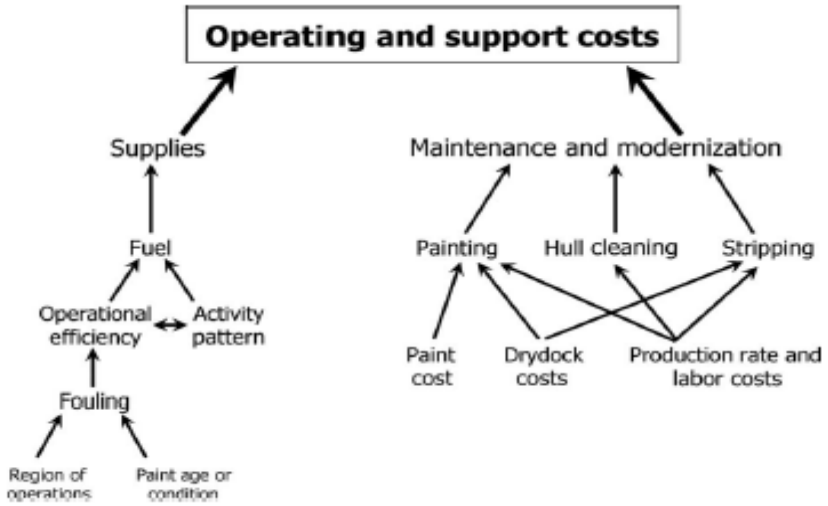

Figure 2 – Operating and support factors that are directly associated with the costs increase in the shipping industry (adapted in Schultza *et al.*, 2011)

In figure 3 it is possible to observe the relation between the annual costs per ship and the fouling rate. This figure shows a linear response until a value of 70 for the fouling rate (Schultza *et al.*, 2011).

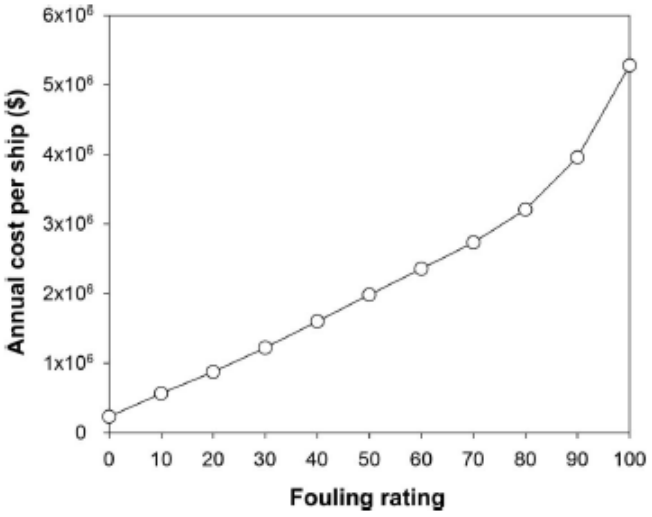

Figure 3 – Annual costs per ship for an increasing value of fouling rate (adapted in Schultza *et al.*, 2011)

By looking into these costs we can conclude that it is necessary to apply antifouling control strategies in order to decrease these values. However, it is important that those methods or strategies do not affect the surrounding environment and the non-target species that live in the marine ecosystem.

2.3. Microfouling and Macrofouling

Marine biofilms can be composed by different types of organisms. Figure 4 shows some examples of those organisms.

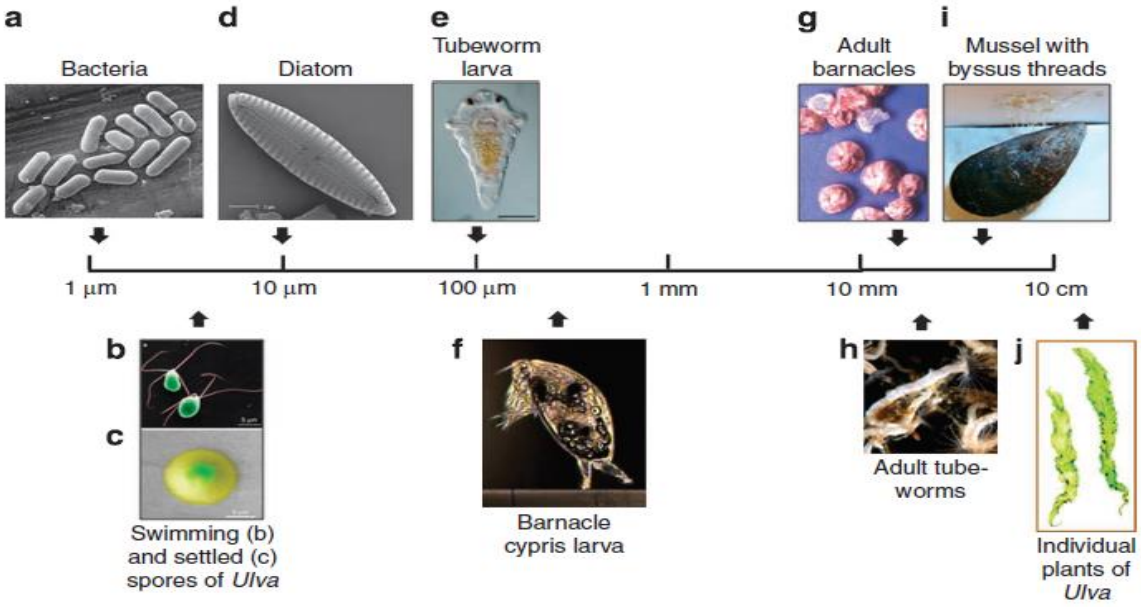

Figure 4 – Different types of marine fouling organisms and size scale comparation (adapted in Callow and Callow, 2002)

Microfouling is caused by bacteria and micro algae which form a complex biofilm. Examples of species that live in those biofilms are *Pseudomonas aeruginosa*, *Alphaproteobacteria*, *Firmicutes*, *Myroids odoratimimus*, *Micrococcus luteus*, *Pseudoalteromonas tunicate* (for bacteria), *Amphora coffeaeformis*, *Navicula directa* (for diatoms) (Inbakandana *et al.*, 2013; Kougo *et al.*, 2012; Wigglesworth-Cooksey and Cooksey, 2005).

Macrofouling refers to the macro-community of organisms which can adhere to the microfouling. Macrofouling can be divided into two categories: “soft fouling” (larger algae and invertebrates, such as soft corals, sponges, anemones, tunicates and hydroids) and “hard fouling” (invertebrates such as barnacles, mussels and tubeworms) (Jakob *et al.*, 2008; Characklis *et al.*, 1982; Lisbeth *et al.*, 2003).

Marine biofouling is a process that consists in three steps. The first one consists in the formation of a conditioning film composed by organic material (proteins and carbohydrates). The second step refers to the adhesion and development of biofilm (by the microorganisms that compose them such as bacteria and microalgae, primarily diatoms – microfouling). The final step is the growth of the biofilm and the adhesion of bigger organisms that form the macrofouling (e.g. barnacles and seaweed) (Jakob *et al.*, 2008). Many factors may influence the adhesion of marine biofilms and fouling to the surface: surface morphology, chemical proprieties of the surface, substratum proprieties, presence of enzymes and metallic oxides, surface roughness and shear stress. In the

case of macrofouling, competition and predation are also important factors (Jakob *et al.*, 2008; Lisbeth *et al.*, 2003; Callow and Callow, 2002; Palacio and Bhushan, 2011; Satheesh and Wesley, 2010).

2.4. Factors Affecting Bacterial Adhesion and Biofilm Formation

Many factors affect bacterial adhesion and biofilm formation, but in this work only the surface properties and shear stress were studied.

It is known that initial attachment is affected by the surface physicochemical properties and topography features (Whitehead and Verran, 2008).

It was shown for bacterial spores (by *Husmark and Ronner et al.*, 1993) that their adhesion increases with the increment of surface hydrophobicity. *Carson and Allsopp et al.* (1980) suggested that on hydrophobic plastics, the cell attachment occurs rapidly and *Dexter et al.* (1979) demonstrated that on hydrophilic surfaces (e.g. metallic oxides) an increase in cells adhesion takes longer times (Whitehead and Verran, 2008).

Biofilms can grow in different types of surfaces. These surfaces can be metals (e.g aluminium, stainless steel, copper) and polymers for example. *Starr and Jones et al.* (1957) demonstrated that some bacteria are able to grow on copper despite its antimicrobial proprieties (Whitehead and Verran, 2008). *Kougo et al.* (2011) investigated the formation of biofilm on seven metallic oxides (WO_3, Fe_20_3, TiO_2, ZnO, CeO_2, Cr_2O_3 and Ag_2O) and concluded that CeO_2, Cr_2O_3 and Ag_2O had higher inhibition capabilities because of the effect of dissolved ions (Kougo *et al.*, 2012). For the cases of polymers, plastic materials are the preferential ones for the bacteria adhesion. *Bachmann and Edyvean et al.* (2006) demonstrated that *Aquabacterium commune* biofilms possessed larger density on medium density polyethylene than on stainless steel (SS) (Whitehead and Verran, 2008).

Dobretsov, Abed and Voolstra et al. performed an experiment to see if the colour of the substrate has any influence in the bacteria adhesion. They found that at the substrate with black colour (coloured acrylic tile) had a higher bacterial adhesion than the white substrate (figure 5) (Dobretsov *et al.*, 2013).

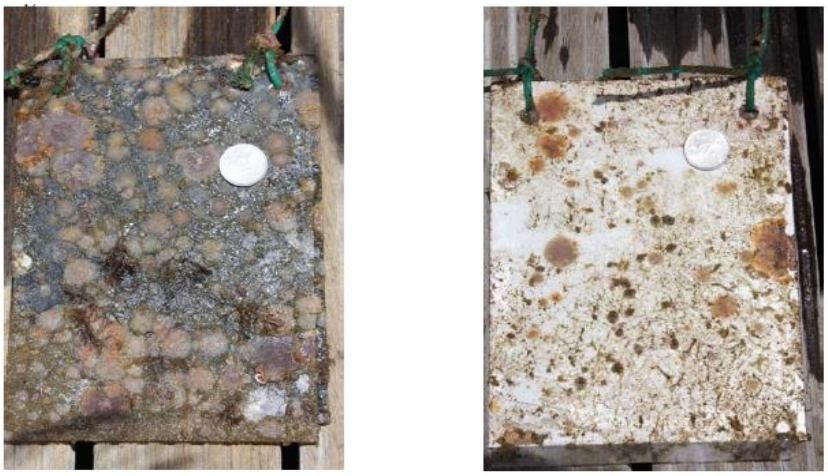

Figure 5 – The resulting fouling on two different coloured substrates after days submerged in sea water (adapted in Dobretsov *et al.*, 2013)

The retention of microorganisms on a surface will also be affected by the surface roughness. The increase of the surface roughness will favour the retention of microorganisms. After biofilm establishment, the surface roughness may also contribute for the macrofouling adhesion by providing more anchorage points (Whitehead and Verran, 2008).

Shear stress is also one of the most important factors which influences the formation of the biofilms. In figure 6 it is represented the different processes resulted from the application of shear stress on biofilms.

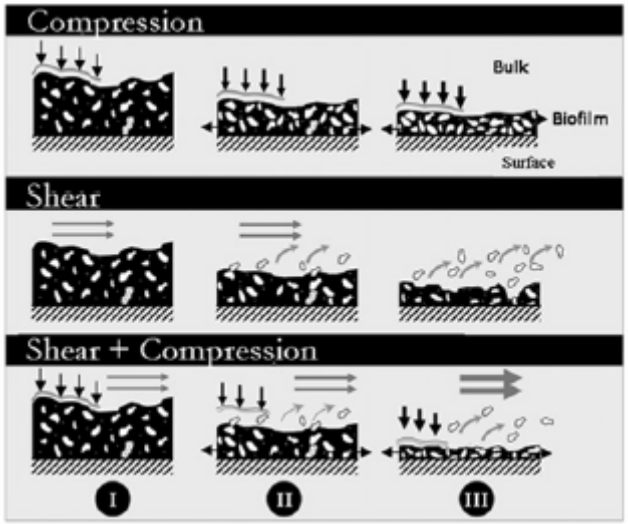

Figure 6 – Effects of shear stress on biofilms (adapted from Paul *et al.*, 2012)

In figure 6, it is demonstrated that the shear effect alone causes the destruction of the outer layers of the biofilm leading to the detachment of the biofilm biomass and with the compression phenomena the biofilm becomes thinner. Several investigations were made through the previous years to support the veracity of this hypothesis. For example, *Christensen and Characklis et al*, in 1990, proved the existence of a linear relation between shear stress and biofilm density (in the range of 1 and 15 Pa) while, in 1998, *Kwok et al.* found that the biofilm density increases with the increase of shear stress (in the same range of shear stress) (Paul *et al.*, 2012). In a more recent study (2012), *Ochoa and Paul et al.* demonstrated by several experiments, with different growth conditions and applied shear stress (example and results in figure 7), that at higher shear stress, the biofilms presented a lower amount of biomass and thickness. They found that for both growth and non-growth test conditions, when shear stress is applied, that biofilm thickness decreases with an increase in biofilm density. This relation leads to the conclusion that the shear stress determines the average thickness of the biofilm. They also observed that for the different growth conditions and the value of shear stress applied, a gradual detachment and deformation of the biofilm occurs and for higher values than 2 Pa, the thickness of the biofilm is governed by the compression phenomena (Paul *et al.*, 2012).

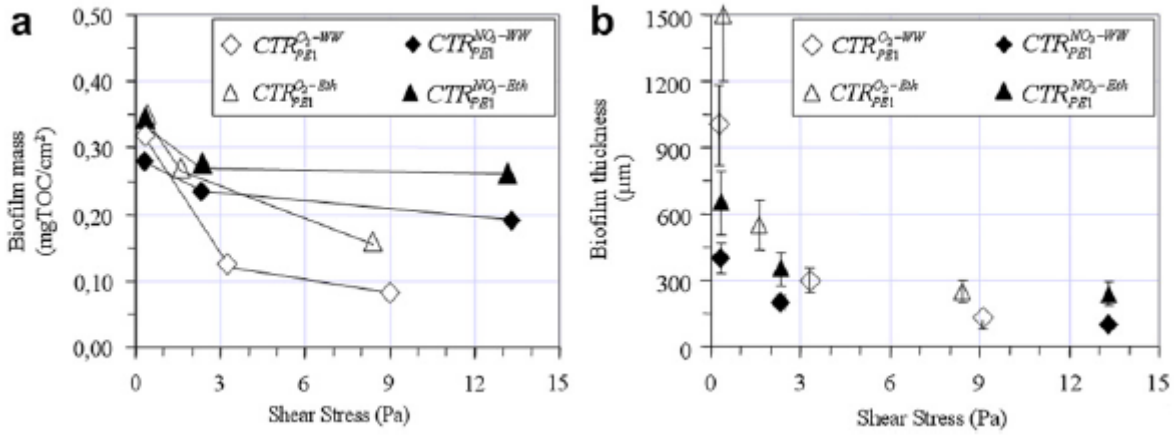

Figure 7 – Biofilm mass and thickness obtained by *Ochoa and Paul et al.* when using different substrates conditions on plastic plates in the Couette Taylor Reactor (CTR) (adapted in Paul *et al.*, 2012)

2.5. Bacterial Adhesion Study

Shear stress and type of surface are two of the most important factors in bacterial adhesion and biofilm formation, therefore, in this study the effect of different shear stresses (which can be found in the marine environment) on the adhesion of a marine bacteria (*Pseudoalteromonas tunicata*) to two different surfaces, stainless steel (which is normally used as core material in naval ships and PVC (used in underwater pipelines) was evaluated. Different *in vitro* platforms can be used to study bacterial adhesion and biofilm formation. There are the flow systems which include flow cell perfusion model, rotating disc, laminar flow system and radial flow chamber, etc. (An and Friedman, 1997) and the high-troughtput platforms such as the microtiter plates which enable testing different conditions at the same time. In this work the 6-well microtiter plate was selected since its enables to test different shear stresses, different surfaces with the help of coupons made of stainless steel or PVC. The enumeration of Colony Forming Units (CFU) and the 4',6-diamidino-2-phenylindole (DAPI) staining protocol allows to determine the quantity of adhered bacteria in the coupons.

In this work, the study of the influence of the action of one biocide on the development of the marine biofilms is also made. The biocide used is known as BDMDAC which is a quaternary ammonium compound (QAC). The QACs are compounds that possess strong antimicrobial properties and weak detergent properties. The BDMDAC is a component of benzalkonium chloride (BAC), extracted from coconut oil. BAC has many applications, manly as disinfectant and sanitizer in hospitals, food plants, homes and other public places. Others include as preservatives and antiseptics in healthcare products (e.g. eyewashes, nasal sprays, hand and face washes, etc.) and as antistatics, emulsifiers and preservatives in the coatings industries (e.g. paints). Due to its safe use (even on human skin) and strong bactericidal properties, it can be concluded that BDMDAC's application on sea won't affect non-target species. To finalize, BDMDAC is a cationic surfactant and has a long carbon chain composed of 12 carbons (Ferreira et al., 2011).

3. Materials and Methods

3.1. Strains and Culture Media

In this work, *Pseudoalteromonas tunicata* (*P. tunicata*) was first inoculated in a sterile vaatanen nine salt solution (VNSS) medium made in a 1000 mL sterile schott (17.6 g/L NaCl; 1.47 g/L Na_2SO_4; 0.08 g/L $NaHCO_3$; 0.25 g/L KCl; 0.04 g/L KBr; 1.87 g/L $MgCl_2.6H_2O$; 0.41 g/L $CaCl_2.2H_20$; 0.008 g/L $SrCl.6H_2O$; 0.008 g/L H_3BO_3; 1.0 g/L peptone; 0.5 g/L yeast extract; 0.5 g/L glucose; 0.01 g/L $FeSO_4.7H_2O$ and 0.01 g/L Na_2HPO_4) and incubated overnight (30 ºC; 120 rpm). A medium with an equal composition plus 15 g/L of agar was made, in a 1000 mL sterile schott, for the CFU plates for the CFU counting method.

A second culture media was also prepared in a 1000 mL schott to be used later in the adhesion assay. This media is the artificial sea water (ASW) composed by 23.4 g/L NaCl; 24.6 g/L $MgSO_4.7H_2O$; 1.5 g/L KCl and 2.9 g/L $CaCl_2.2H_2O$.

In the final experiments, another media was used also in the adhesion assay: natural sea water (NSW), sterilized by a filtration method using a 0.2 µm pore membrane. The NSW used in the adhesion assays was collected from Praia da Agudela (Matosinhos, Portugal).

3.2. Growth Rate Curve Assay

In order to determine the bacterial growth rate, bacterial growth was followed for 8h. An aliquot from the overnight culture was inoculated in into VNSS (starting with an OD of 0.1) in the same conditions as the overnight. Samples were aseptically retrieved every 30 minutes. The *P. tunicata* growth was determined by measuring the optical density (OD) of the samples at 600 nm in a spectrophotometer.

3.3. BDMDAC MIC Pre-Test Assay

3.3.1. Biofilm preparation

In order to find the Minimum Inhibitory Concentration (MIC) of BDMDAC several concentrations were tested: 1000; 500; 250; 125; 62.5; 31.25; 15.62; 7.813; 3.91; 1.95 µg/mL and SS surfaces were used as substratum. Benzyldimethyldodecylammonium

chloride (BDMDAC – molecular weight of 339.9) was obtained from Fluka (Portugal). Each BDMDAC solution was prepared from a stock solution (1mg/ML) with sterile ASW medium before each experiment.

From the overnight culture, two aliquots were centrifuged (at 3202 g for 10 min at 25ºC) to separate the bacterial cells from the supernatant. A second centrifugation was made after cell resuspension in the ASW media. After the centrifugations, the *P. tunicata* was resuspended again with the ASW media to a final OD of 0.1 (optical density measured in a spectrophotometer at 600 nm).

The cellular suspension was used to inoculate the wells of a 12-well microtitter plate containing a SS coupon. Sterile ASW media was used as negative control. Plates were incubated overnight at 25ºC. Experiments were made in triplicate.

3.3.2. MIC determination

After the overnight biofilm formation, the microtiter plates were washed with 8.5% NaCl solution, and then 2 mL of each BDMDAC concentration solution were added to each well. The plates were incubated overnight at 25ºC again.

In the next day, one wash was made and then the coupons were placed in sterile 15 ml tubes with 9 ml of 8.5% NaCl solution and vortexed. Suspensions were further diluted prior to plating (up to 10^{-4}). And the CFU plates were incubated overnight and counted.

3.4. Bacterial Biofilm Assay

3.4.1. Biofilm Preparation

The steps used in this subsection are similar to the subsection 3.3.1 with exception of the microtiter plate type and organization. In this assay, three 6-wells microtiter plates were used with the following scheme: 3 wells with 1 SS coupon and 3 wells with 1 PVC coupon for each well (see figure 8). One plate was used for control (corresponding to 24 hours of incubation), another for 1 hour of treatment with BDMAC and the last one for 3 hours of treatment with the biocide. Experiments were made in triplicate.

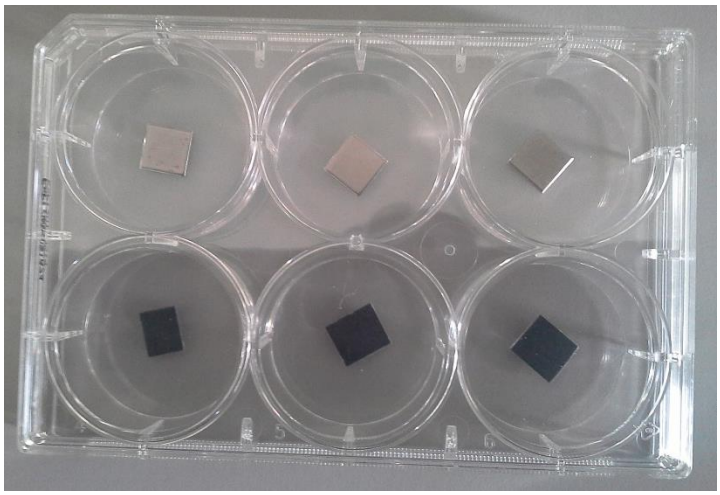

Figure 8 – Scheme of the 6-wells microtiter plate with coupons used in the assays

3.4.2. BDMAC Treatment

The MIC determined to BDMDAC was 10 µg/mL and two values of shear stress were used (0.1 and 1 Pa).

After the overnight biofilm formation (25ºC, 40 rpm for 0.1 Pa and 185 rpm for 1 Pa), the plates were washed with 8.5% NaCl solution. The coupons placed in one of the microtiter plates were the control. To the remaining two microplates a 4 mL of 10 µg/mL BDMAC solution were added to each well and incubated for 1 hour and 3 hours respectively (25ºC). After cell suspension, plating and incubation (as described before) CFU enumeration was performed.

In the BDMAC assays, only the ASW was used during the biofilm formation step.

In addition, two assays were conducted in similar way but only biofilm controls were made without the use of the biocide, using both ASW and NSW. The biofilms were let to grow during 24, 25 and 27 hours. Experiments were made in triplicate and standard deviations determined for each condition.

3.5. DAPI Staining Assay

3.5.1. Biofilm Preparation

Biofilms were formed as described in subsection 3.3.1 and the microplates have the same as the scheme presented in subsection 3.4.1 with the exception that only biofilm controls

were used (24, 25 and 27 hours of biofilm incubation, each time of incubation for one microplate).

3.5.2. DAPI Staining

After overnight biofilm formation (25ºC, 40 rpm for 0,1 Pa and 185 rpm for 1 Pa), the microplates (retrieved from the incubator at each respective time) and the wells were washed once. After that, the coupons were placed on microscope slides. Then, in dark environment, 1 drop of DAPI was added to each coupon and placed to rest during 10 minutes. The excess liquid was removed, and the microscope slides were putted stored at 4ºC to be visualized later (maximum one day) on the microscope (Nikon digital sight DS-RI 1, Japan). Photos of each coupon were taken, in order to determine the number of adhered bacterial cells in each coupon.

Four different experiments were made, two assays using ASW and the remaining two using NSW under low (0.1 Pa) and high shear stress (1 Pa). Experiments were made in triplicate.

4. Results and Discussion

4.1. Growth Curve Rate Assay

Figure 9 represents the growth curve for *P. tunicata* over the 8 hour period.

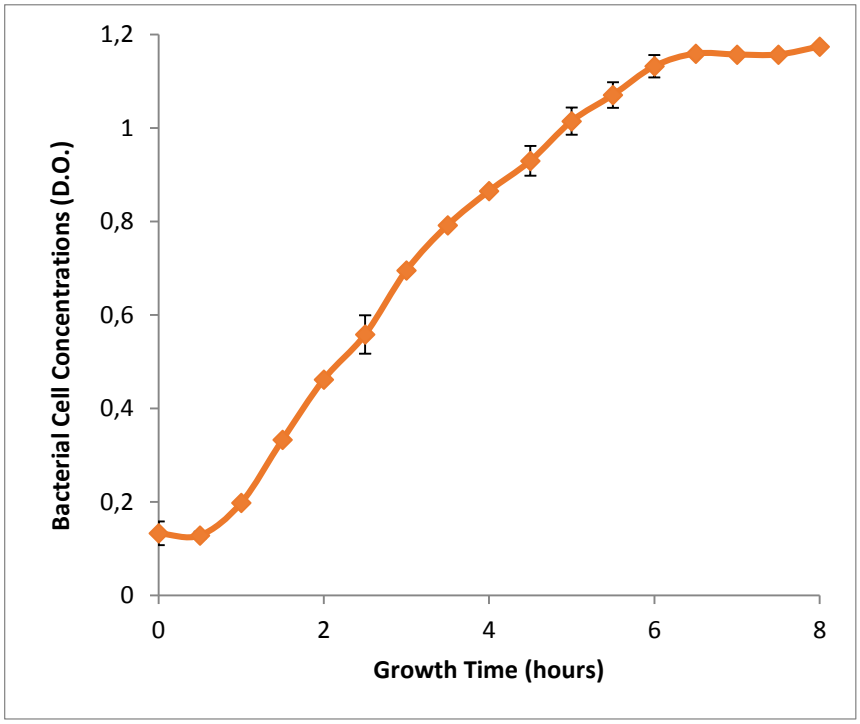

Figure 9 – *P. tunicata* growth curve (standard deviation between the duplicates is represented and the optical densities were measured at 600 nm in a spectrophotometer)

It is possible to observe that the growth curve has 3 distinct phases. The lag phase (start point of the bacterial growth) lasts for only half of hour leading to the exponential phase during 6 hours (rapid growth) and stabilizes at 6.5 h point. At that time, the stationary phase begins (bacterial growth reaches a limit). For that reason, the Michaelis-Menten law principles can be applied in order to calculate the value of the specific growth rate which it is equal to 0.533 h^{-1}.

4.2. BDMDAC MIC Pre-Test Assay

Figure 10 shows the logarithmic number of *P. tunicata* adhered cells to SS for each BDMDAC concentration (concentrations higher than 65.2 µg/mL were not considered because no bacterial growth was observed).

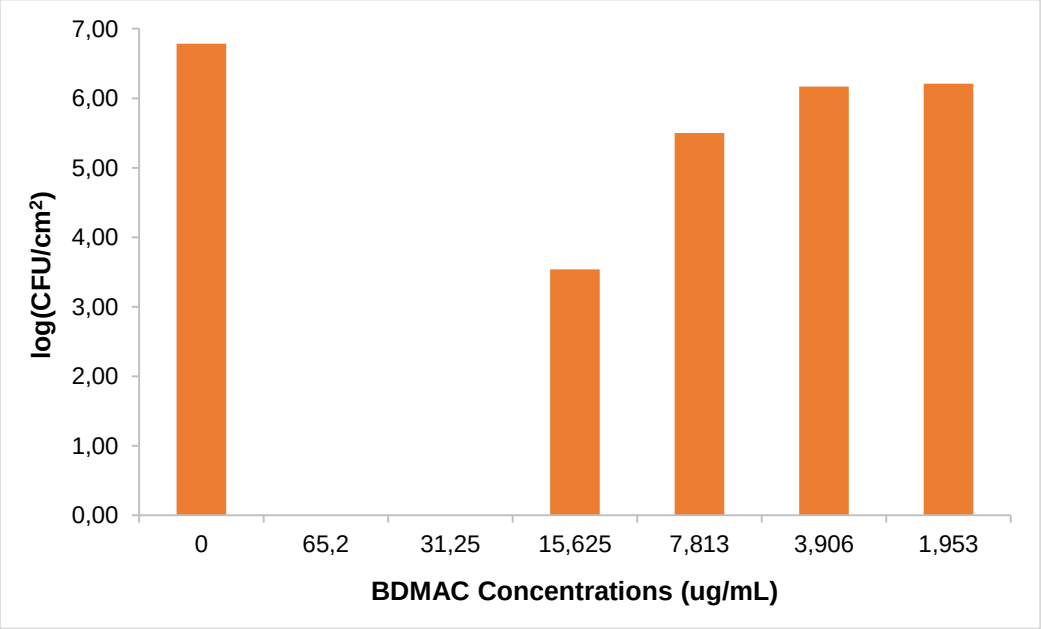

Figure 10 – MIC of BDMDAC (using cultures of *P. tunicata* adhered cells to SS)

The figure indicates that for a concentration of 31.25 µg/mL, no bacteria has survived. The MIC of BDMDAC must be between the values of 15.625 and 7.813 µg/mL. A BDMDAC concentration of 10 µg/mL was considered as the MIC for the further experimental work of the thesis. The Minimum Bactericidal Concentration (MBC) is between 31.25 and 15.625 µg/mL.

4.3. Bacterial Cells Adhesion Assay

The purpose of this method was to test the efficiency of biofilm elimination by BDMDAC under determined hydrodynamic conditions. For a shear stress value of 0.1 Pa in ASW medium, the following results were obtained from SS (Figure 11) and PVC (Figure 12).

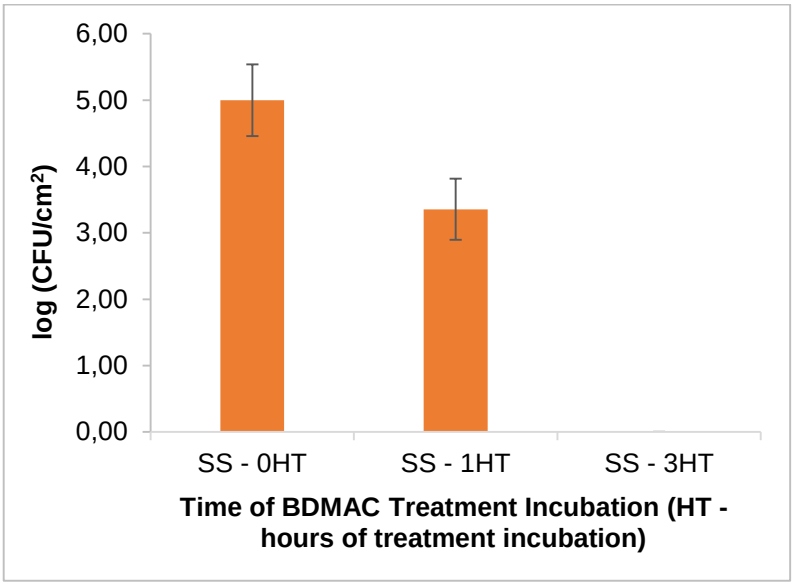

Figure 11 – Influence of BDMDAC MIC solution on *P. tunicata* adhered cells number on SS surface (0.1 Pa and ASW medium)

In the figure 11, SS – 0HT represents the biofilm control of 24 hours incubation time. It shows that the BDMAC managed to kill a large part of the cells (2 log) in just one hour of exposure. Then at 3 hours of incubation, all bacteria were dead.

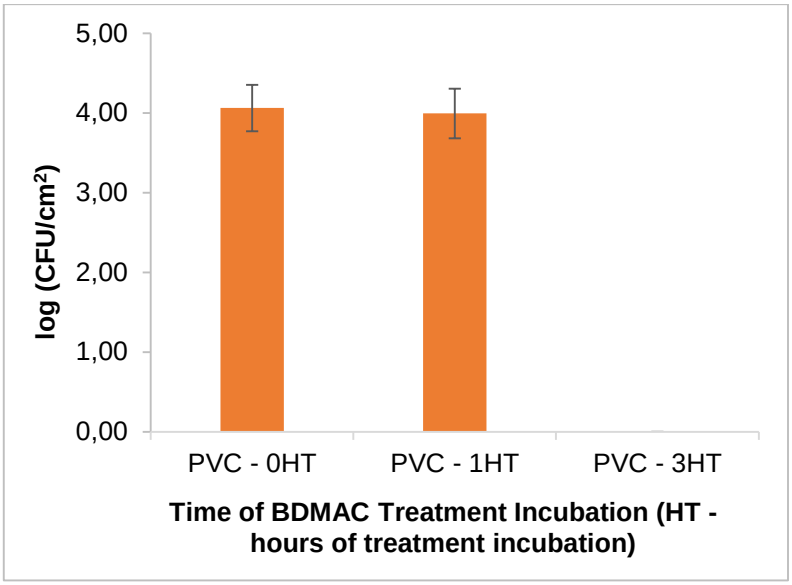

Figure 12 – Influence of BDMDAC MIC solution on *P. tunicata* adhered cells number on PVC surface (0.1 Pa and ASW medium)

It seems that at 24 hours of incubation the biofilm adhered on PVC had less cells than the biofilms that developed on the SS surface. However, after 1 hour of exposure to the biocide there were more cells in PVC than in SS indicating that biofilms developed on PVC surfaces may be more resistant than the biofilms grown on SS. Although, at 3 hours, all cells were dead similarly to what was observed for SS.

Figures 13 and 14 shows the percentage of biofilm reduction in the cases presented up until now.

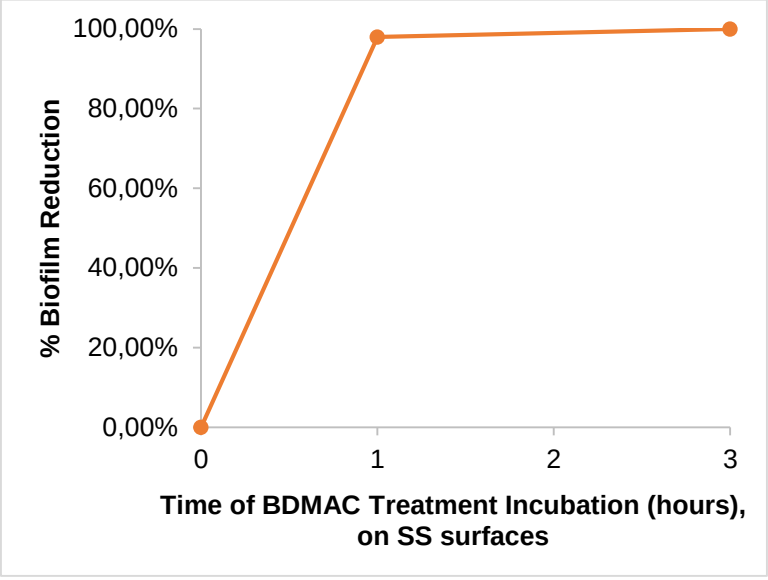

Figure 13 – Percentage of biofilm reduction in the three hours of BDMDAC treatment on SS Surfaces (0.1 Pa and ASW medium)

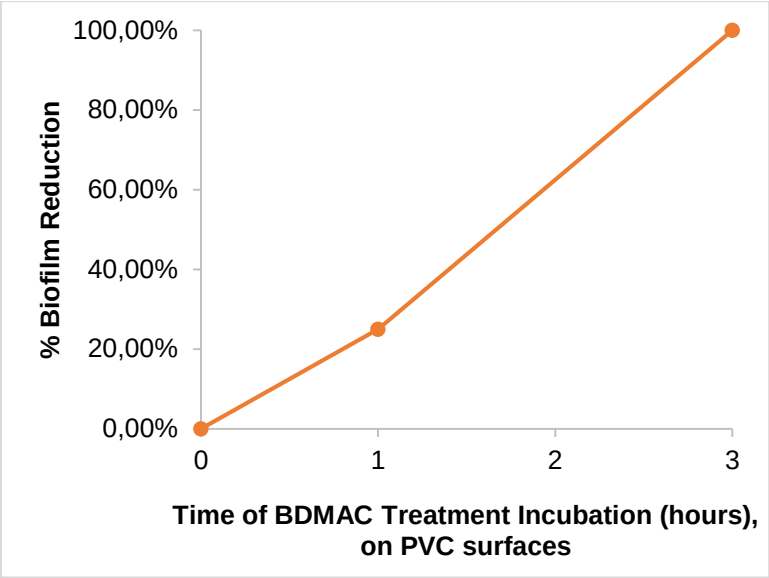

Figure 14 – Percentage of biofilm reduction in the three hours of BDMDAC treatment on PVC Surfaces (0.1 Pa and ASW medium)

The same experiment was repeated using a shear stress of 1 Pa and the same medium.

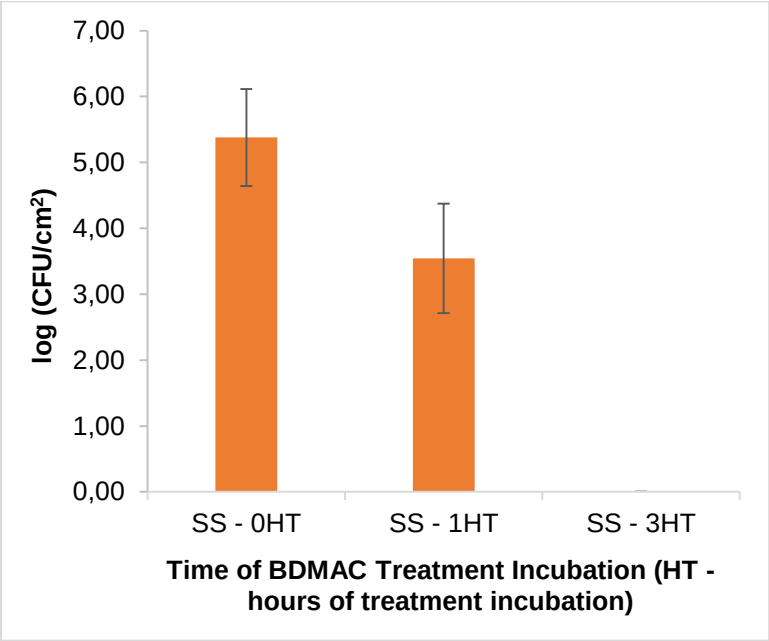

Figure 15 – Influence of BDMDAC MIC solution on *P. tunicata* adhered cells number on SS surface (1 Pa and ASW medium)

If the figure 15 is compared with figure 11, it is concluded that in the higher value of shear stress (1 Pa), higher amounts of biofilm were formed at this shear stress and similarly to what was observed for the lower shear stress (0.1 Pa), a 2 log reduction was also

obtained after 1 hour of BDMDAC treatment and total biofilm killing was observed after 3 hours.

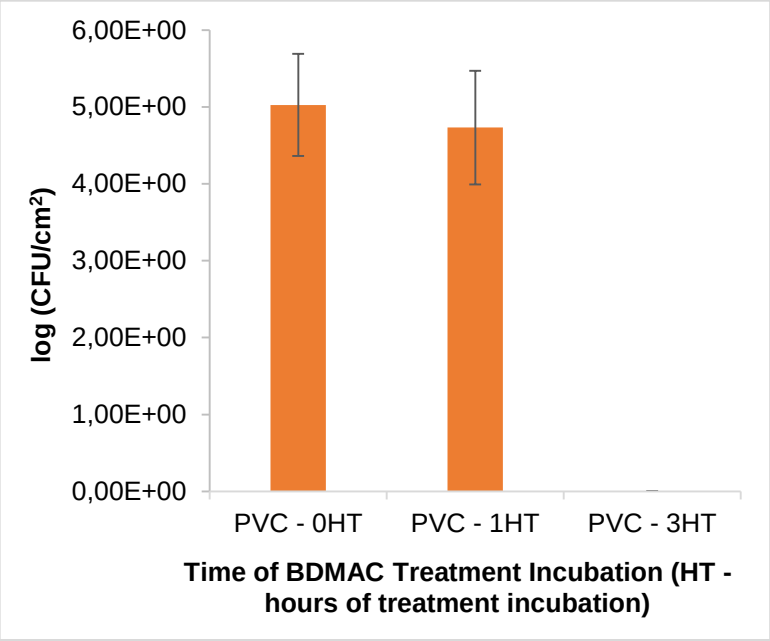

Figure 16 – Influence of BDMDAC MIC solution on P. tunicata adhered cells number on PVC surface (1 Pa and ASW medium)

Similarly to what was observed for the lower shear stress, the biofilms formed on PVC seem more resistant than those formed on SS and the biofilm formation was also enhanced. After 3h the biofilm was dead.

Figures 17 and 18 present the percentage reduction of biofilm.

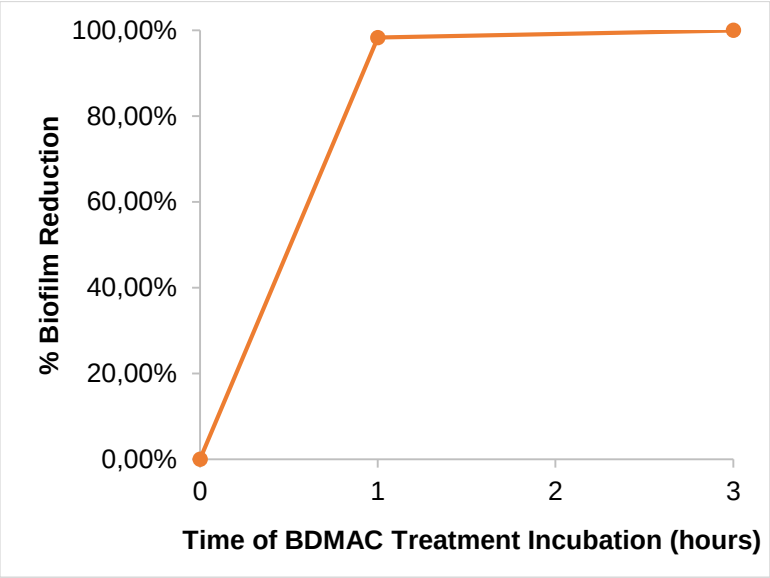

Figure 17 - Percentage of biofilm reduction in the three hours of BDMDAC treatment on PVC Surfaces (1 Pa and ASW medium)

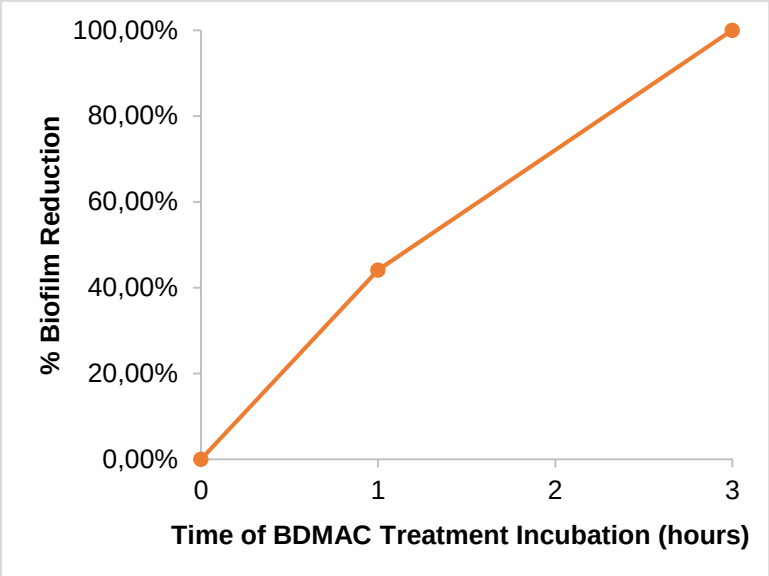

Figure 18 - Percentage of biofilm reduction in the three hours of BDMDAC treatment on PVC Surfaces (1 Pa and ASW medium)

In the case of the biofilms developed on PVC, at high shear stress values the biofilm reduction is even faster, meaning that the resistance of this kind of biofilms may be decreased with the increment of the shear stress value.

From these two experiments (ASW medium) the following observations were made:

Biofilm formation seemed to be enhanced on stainless steel when compared to PVC at low shear stress.

A higher shear stress seems to have increased biofilm formation and similar amounts of biofilms seemed to have formed on both surfaces.

Shear stress did not seem to affect biofilm resistance for the first 3h of treatment with BDMDAC in SS but biofilm resistance in PVC seemed to decrease.

Give the initial results, we decided to test biofilm formation in longer time intervals (24h and above) and verify if the biofilm reduction was due to the effect of BDMDAC or by another factor. In this case, no BDMDAC was introduced on the wells. The following results were obtained (Figures 19 and 20).

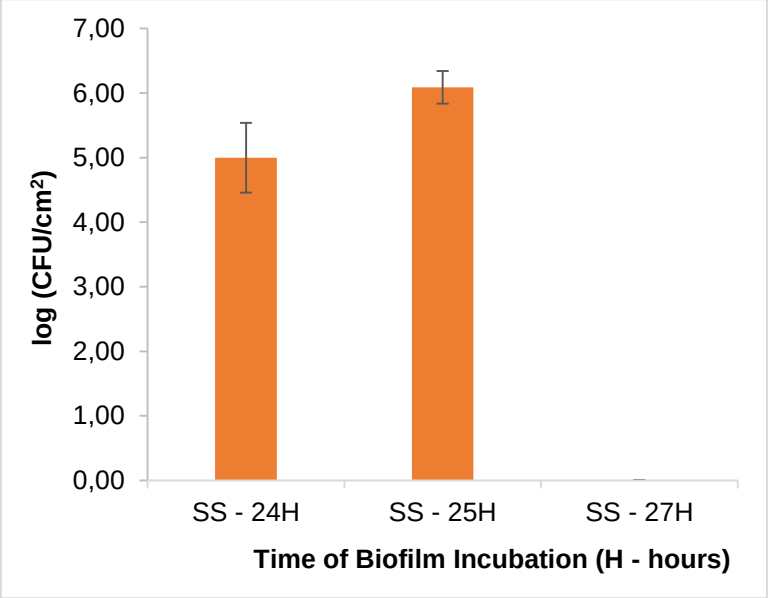

Figure 19 – *P. tunicata* adhered cells number on SS surface (0.1 Pa and ASW medium)

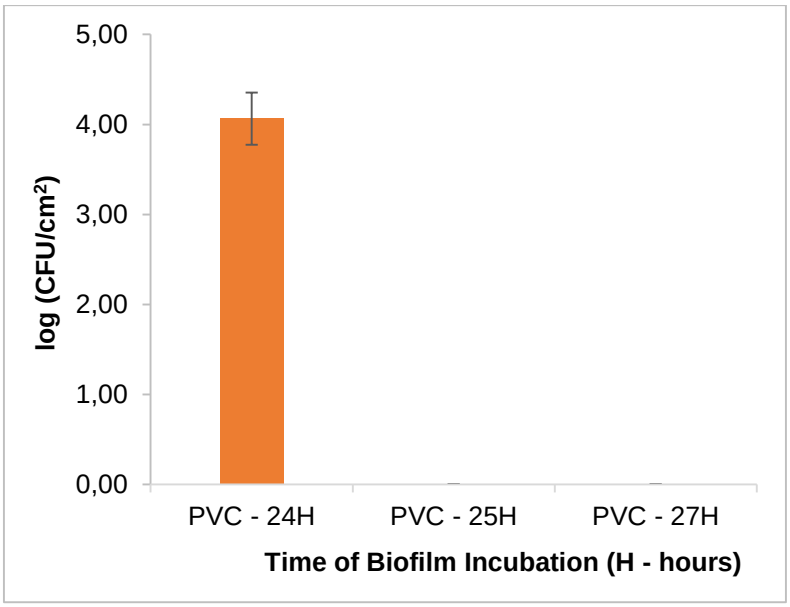

Figure 20 – *P. tunicata* adhered cells number on PVC surface (1 Pa and ASW medium)

These results are strange as it seems that after 25h for SS and 24h for PVC the biofilm is completely dead.

This leads to question if it was really the influence of the biocide that led to removal of the biofilm or it was for another reason. Since the results presented by figures 19 and 20 did not make sense, several hypothesis were discussed to examine the results obtained until this point. After discussion, it was proposed that the cells maybe were not efficiently removed by the vortexing method. So the experiment was repeated but this time all cell suspensions (well supernatant, washing solution, resuspension solution) were analysed by DAPI staining as well as the surface after the vortexing procedure to make sure that the cells were being efficiently removed or if the cells remained adhered on the coupons.

4.4. DAPI Assay

In this assay four experiments were made: two for each medium then two for each shear-stress value. Figure 21 represent the results originated from those four experiments.

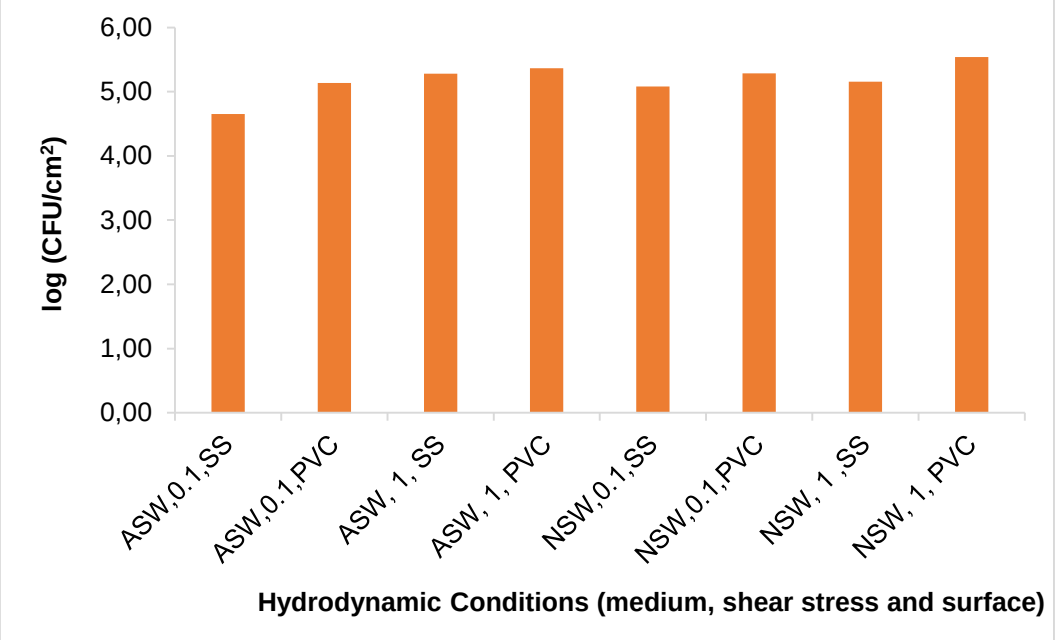

Figure 21 – *P.tunicata* adhered cell numbers of biofilms grown on diverse hydrodynamics conditions (medium, shear stress, surface type)

By observing figure 21, it was concluded that the cells were not removed from the coupons indicating that for prolonged incubations the adhesion forces between the cells and the surface were higher than the detachment forces induced by the vortexing procedure. Reanalysing figure 19, it is interpreted that at 24 and 25 hours of incubation, for SS, the vortexing method was able to remove cells but at 27 hours, the cohesion forces of the biofilm were already strong enough to surpass the applied detachment forces, so no cells were removed. For biofilms developed on PVC (figure 20), after 25 hours of incubation, no cells were able to be retired from the surface. This leads to the conclusion that the biofilms formed on PVC possess stronger adhesion to the surface than the biofilms developed on SS. This procedure had been previously tested and optimized for another Gram negative bacteria, *Escherichia coli* using the same surface materials (Gomes *et al.*, 2014). On that instance, the extent of cell removal from the surface due to vortexing was assessed and it was found to be greater than 95% (Gomes

et al., 2014). Thus, the results presented on the previous section can be interpreted as a measurement of biofilm cohesion on different materials upon BDMDAC treatment.

5. Conclusions and Future Outlook

5.1. Conclusions

Marine biofouling on man-made structures is costly and the US Navy alone spends about $1 billion per year in fouling associated expenditures. Therefore it is necessary to develop antifouling strategies to reduce those costs. However it is important that those strategies do not affect the surrounding environment where they are applied. In order to fulfil that challenge, it is fundamental to understand the basics of the formation of marine biofilms to find a method to delay the growth of the biofilm.

One approach, it is to study the factors that influence the onset of marine biofilms. One of them is the shear stress and the type of surface where the microorganisms adhere and both factors were addressed in this work.

Initial findings with a chemical biocide indicated that biofilm formation seemed to be enhanced on stainless steel when compared to PVC at low shear stress. Also, a higher shear stress seemed to have increased biofilm formation and similar amounts of biofilms seemed to have formed on both tested surfaces. Additionally, shear stress did not seem to affect biofilm resistance for the first 3h of treatment with BDMDAC in SS but biofilm resistance in PVC seemed to decrease. Later it was discovered that the biofilm detachment procedure by vortexing was not efficient for this particular bacterial strain. This is a somewhat puzzling result as the detachment method had been optimised for another Gram negative bacteria (*E. coli*) forming biofilms in the same surfaces (Gomes *et al.*, 2014). Despite the high removal efficiencies obtained on that work (approximately 95%) it seems that *P. tunicata* formed a much more cohesive biofilm under the conditions tested. Thus, the initial results that were obtained are probably not indicative of the biofilm resistance to the biocide but rather about the biofilm cohesion upon chemical treatment. In any case, given the uncertainty in the control of the shear forces exerted during vortexing, these initial results must be observed with caution. The fact that *P. tunicata* apparently formed more cohesive biofilms than *E. coli* in very similar conditions may be attributed to differences in the cell wall composition, the effect of cellular appendages or the amount and composition of the EPS formed but given the time constraints of this project these issues were not addressed.

5.2. Future Outlook

As highlighted in the previous section, it seems that the biofilms formed by *P. tunicata* on the tested materials are characterised by a strong cohesion. The determinants of the cohesion such as the amount and composition of the EPS formed as well as the influence of the hydrodynamic conditions and surface properties on biofilm cohesion should be addressed on future studies.

References

Barbara Wigglesworth-Cooksey, Keith E. Cooksey; Use of Fluorophore-Conjugated Lectins To Study Cell-Cell Interactions in Model Marine Biofilms; Applied And Environmental Microbiology; 71 (2005); 428–435

C. Ferreira, A. M. Pereira, M. C. Pereira, L. F. Melo and M. Simões; Physiological changes induced by the quaternary ammonium compound benzyldimethyldodecylammonium chloride on Pseudomonas fluorescens; Journal of Antimicrobial Chemotherapy; (2011); 1 – 8

Callow, J. A., Callow, M. E.; Trends in the development of environmentally friendly fouling-resistant marine coatings (2011)

D. Inbakandana, C. Kumara, L. Stanley Abrahama, R. Kirubagaranb, R. Venkatesanb, S. Ajmal Khanc; Silver nanoparticles with anti microfouling effect: A study against marine biofilm forming bacteria; Colloids and Surfaces B: Biointerfaces; 111 (2013); 636 – 643

E. Paul, J. C. Ochoa, Y. Pechaud, Y. Liu, A. Liné; Effect of shear stress and growth conditions on detachment and physical properties of biofilms; Water Research; 46 (2012); 5499 – 5508

Gomes L. C., Silva L.N., Simões M., Melo L.F., Mergulhão F. J.; Escherichia coli adhesion, biofilm development and antiobiotic susceptibility on biomedical materials; Society for Biomaterials; 103A (2014); 1414 – 1423

Hans-Curt Flemming, Jost Wingender; The biofilm Matrix; Nature Reviews, Microbiology; 8 (2010); 623 – 633

Hilary M, Lappin-Scott, J William Costerton; Bacterial biofilms and surface fouling; Biofouling; 1 (1989); 323 – 342

Jakob Broberg Kristensen, Rikke Louise Meyer, Brian Søgaard Laursen, Stepan Shipovskov, Flemming Besenbacher, Charlotte Horsmans Poulsen; Antifouling enzymes

and the biochemistry of marine settlement; Biotechnology Advances; 26 (2008); 471 – 481

Lisbeth R. Hilberta, Dorthe Bagge-Ravnb, John Koldc, Lone Gramb; Influence of surface roughness of stainless steel on microbial adhesion and corrosion resistance; International Biodeterioration & Biodegradation; 52 (2003); 175 – 185

Maureen E Callow and James A Callow; Marine biofouling: a sticky problem; Biologist; 49 (2002); 1 – 5

Manuel L. B. Palacio, Bharat Bhushan; Bioadhesion: a review of concepts and applications; The Royal Society; 370 (2011); 2321 – 2347

M. P. Arpa-Sancet, C. Christophis, A. Rosenhahn; Microfluidic Assay to Quantify the Adhesion of Marine Bacteria; Biointerphases; 7 (2012); 1 – 9

M.P. Schultza, J.A. Bendickb, E.R. Holmb, W.M. Hertel; Economic impact of biofouling on a naval surface ship; 27 (2011); 87 – 98

T. Kougo, D.Kuroda, N.Wada, H.Ikegai, H.Kanematsu; Biofouling of various metal oxides in marine environment; Journal of Physics; 352 (2012); 1 – 5

Sergey Dobretsov, Raeid M. M. Abed, Christian R. Voolstra; The effect of surface colour on the formation of marine micro- and macro-fouling communities; Biofouling journal; 1 – 37

S. Satheesh, S. Godwin Wesley; Influence of substratum colour on the recruitment of macrofouling communities; Journal of the Marine Biological Association of the United Kingdom; 90 (2010); 941 – 946

W. G. Characklis, M. G. Trulear T, J. D. Bryers Z, N. Zelver; Dynamics Of Biofilm Processes: Methods; Water Resources; 16 (1982); 1207 - 1216

Yuehuei H. An, Richard J. Friedman; Laboratory methods for studies of bacterial adhesion; Journal of Microbiological Methods; 30 (1997); 141–152

K. A. Whitehead, J. Verran; The Effect of Substratum Proprieties on the Survival of Attached Microorganisms on Inert Surfaces; Marine and Industrial Biofouling; (2008); 13 – 33