Universidade do Minho
Escola de Engenharia

Lúcia da Conceição Diogo Chaves Simões

Microbial interactions on biofilm formation by drinking water autochthonous microorganisms

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Microbial interactions on biofilm formation by drinking water autochthonous microorganisms

Doctoral Dissertation for PhD degree in
Chemical and Biological Engineering

Supervisor:
Professora Doutora Maria João Vieira

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É AUTORIZADA A REPRODUÇÃO PARCIAL DESTA TESE APENAS PARA EFEITOS
DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO INTERESSADO, QUE A TAL SE
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A tese de doutoramento aqui apresentada foi suportada por uma bolsa de doutoramento financiada pela Fundação para a Ciência e Tecnologia (FCT) com a referência **SFRH/BD/31661/2006,** financiada pelo Programa Operacional Potencial Humano-Quadro de Referência Estratégico Nacional (POPH – QREN) - Tipologia 4.1 - Formação Avançada, comparticipado pelo Fundo Social Europeu (FSE) e por fundos nacionais do Ministério da Ciência, Tecnologia e Ensino Superior (MCTES).

"*Preferi a ciência ao fino ouro,*
pois a Sabedoria vale mais que
as pérolas e jóia alguma pode igualá-la".

(Prov 8, 10-11)

A toda a minha família

"There is no question that our health has improved spectacularly in the past century. One thing seems certain: It did not happen because of improvements in medicine, or medical science, or even the presence of doctors, much of the credit should go to the plumbers and sanitary engineers of the western world."

Lewis Thomas (speech, 1984)

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ABSTRACT

The provision of safe drinking water (DW) is a top priority issue in any civilized society. Safe DW is a basic need to human development, health and well-being. The main challenge to the DW industry is to deliver a product that is microbiologically and chemically safe, aesthetically pleasing and adequate in quantity and delivery pressure. Normally, the water that leaves a treatment station has quality, but its quality decreases along the travel in the drinking water distribution systems (DWDS). It is well known that biofilms constitute one of the major microbial problems in DWDS that most contributes to the deterioration of water quality. However, their elimination from DWDS is almost impossible, but several aspects can be manipulated in order to prevent and control their growth.

The main goal of this work is to provide a contribution to better understand the important biological and ecological mechanisms (adhesion, coaggregation, microbial diversity and interactions, chemical resistance) involved in biofilm formation in DWDS, with intent to control and prevent their formation, in order to improve DW quality. Thus, several aspects were studied namely: biofilm formation by DW autochthonous bacteria using bioreactors (effects of hydrodynamic conditions and support material); isolation and identification of the autochthonous bacteria present in local DW; studies on their adhesion and biofilm formation abilities (effects of bacteria and support material surface physicochemical properties); studies of coaggregation abilities and bacterial interspecies interactions involved in biofilm formation; detection of microbial secondary metabolites with antimicrobial and biofilm control properties; study on single and multispecies biofilm control with sodium hypochlorite (SHC) and microbial metabolites.

The study of DW biofilm formation was performed in two distinct bioreactors, flow cell and Propella^{TM}, in order to study the influence of hydrodynamic conditions (turbulent and laminar flow) and support material (PVC and SS) on biofilm formation. The biofilms were monitorized over time in terms of total and cultivable bacteria. All the process conditions allowed the formation of biofilms. The biofilm formation in turbulent flow was similar in both bioreactors, regardless the adhesion surface tested. Under laminar flow, the Propella™ bioreactor allowed higher biofilm formation than the flow cell system. The biofilm formation in flow cell systems was higher on PVC surfaces than on SS. While, in Propella^{TM} system biofilm formation on PVC and SS was similar for both flow regimes. This work also allowed the isolation of distinct DW bacteria, which were identified; some of them were selected for the subsequent studies.

Selected DW-isolated bacteria (25 phenotypically distinct) and some support materials commonly used in DW networks (SS304, SS316, copper, PP, PE, silicone and glass) were characterized in terms of surface properties (surface tension and hydrophobicity) by the contact angles measurement and their adhesion potential was studied by means of thermodynamic theory and by adhesion assays in microtiter plates. All bacteria were classified as hydrophilic and the materials as hydrophobic. SS304, copper, PP, PE and silicone thermodynamically favoured adhesion for the majority of the tested strains, whilst adhesion was generally less thermodynamically favourable for SS316, PVC and glass. Studies of adhesion shown that in addition to surface properties, biological characteristics and mechanisms can be involved in the early adhesion processes and may play a determinant role on the adhesion ability. Consequently, the study of adhesion only based on thermodynamic theory did not provide accurate and reliable results. This work also suggests that strongly adherent bacteria (*A. calcoaceticus*) may play a determinant role in primary colonization of surfaces and possibly on the initial biofilm formation in the real environments.

Studies of adhesion and biofilm formation by six selected DW bacteria (*A. calcoaceticus*, *B. cepacia*, *Methylobacterium* sp., *M. mucogenicum*, *Sph. capsulata* and *Staphylococcus* sp.) were performed in order to characterize the relationships between the two phenomena. The adhesion was assessed by the methods referred above and biofilm formation was developed over time in microtiter plates. Adhesion and biofilm formation abilities

were only correlated in the early stages of biofilm formation (24 h). For longer periods (48 and 72 h) some bacteria classified as non-adherent produced large amounts of matured biofilms. Initial adhesion did not predict the ability of the tested DW bacteria to form a mature biofilm, suggesting that other events such as phenotypic and genetic switch during biofilm development and the production of EPS may play a significant role on biofilm formation and differentiation. *A. calcoaceticus*, *Methylobacterium* sp. and *M. mucogenicum* were those bacteria with the ability to produce more biofilm.

Intergeneric coaggregation ability between DW bacteria was studied (visual and microscopy methods) as well as the surface associated molecules (sugars and proteins) involved in this process. Also, the role of bridging bacteria in multispecies biofilm formation in microtiter plates was assessed. The results demonstrate that only *A. calcoaceticus* autoaggregated and coaggregated with four of the five tested bacteria (*Methylobacterium* sp. as exception). These cell-to-cell adhesion mechanisms were mediated by lectin-saccharide interactions. *A. calcoaceticus* exhibited a putative bridging function in multispecies biofilm formation, being their presence in biofilms a colonization advantage.

Single and dual species biofilm formation, and specific activities, by DW bacteria were determined using microtiter plates over time in order to study the role of bacterial interactions on biofilm formation. A series of planktonic studies (assessing the bacterial growth rate, motility and production of quorum sensing inhibitors - QSI) were also performed in order to try to identify key attributes regulating microbial interactions. Evidences of synergy/cooperation, antagonism and neutral interactions were found between DW bacteria. *B. cepacia* had the highest growth rate and motility, and produced QSI. *Methylobacterium* sp., *Sph. capsulata* and *Staphylococcus* sp. also produced QSI. Only for *Sph. capsulata-M. mucogenicum*, *Sph. capsulata-A. calcoaceticus* and *M. mucogenicum-Staphylococcus* sp., dual biofilm formation seems to be regulated by the QSI produced by *Sph. capsulata* and *Staphylococcus* sp. and by the increased growth rate of *Sph. capsulata*.

The chemical control of single and multispecies biofilms with SHC was studied in microtiter plates. The ability of biofilms to recover from disinfection was also assessed. *Methylobacterium* sp. and *M. mucogenicum* single species biofilms had the highest resistance to SHC, while *Staphylocooccus* sp. and *A. calcoaceticus* formed the most susceptible biofilms. In general, multispecies biofilms were more resistant to inactivation and removal than single biofilms. Multispecies biofilms with all the six bacteria had the highest resistance to SHC, while those without *A. calcoaceticus* were the most susceptible. Only biofilms without *A. calcoaceticus* were not able to recover their biomass from the SHC treatments. *A. calcoaceticus* has a key role in the resistance and functional resilience of DW biofilms (its presence in multispecies biofilms increase their resistance and their ability to recover).

The effects of metabolite molecules on single and multispecies biofilm formation were also studied using microtiter plates. Moreover, some bacterial physiological aspects regulating interspecies interactions (planktonic and sessile growth rates, antimicrobial activity, production of QSI, QS molecules and iron chelators) were characterized with the intent to identify bacterial species with biocontrol potential. *A. calcoaceticus*, *B. cepacia*, *Methylobacterium* sp. and *M. mucogenicum* single species biofilms were strongly inhibited by the cell-free supernatants from the other bacteria. *M. mucogenicum* and *Sph. capsulata* cell-free supernatants demonstrated a high potential to inhibit the growth of counterpart biofilms. For multispecies biofilms only cell-free supernatants produced by *B. cepacia* and *A. calcoaceticus* had no inhibitory effects (caused potentiation) on biofilm formation. Multispecies biofilms were highly susceptible to metabolite molecules in the absence of *A. calcoaceticus*.

In conclusion, the work developed in this thesis clearly reveals that *A. calcoaceticus* had the highest ability to adhere to surfaces, coaggregated with partner bacteria and provided resistance and resilience to control conditions. The presence of this bacterium in the tested consortium represented a significant colonization advantage. It seems strategic to consider the presence of this bacterium in the local DW system as a predictor of the presence of SHC resistant biofilms. Also, microbial species diversity association and interspecies interactions increased multispecies biofilm resistance and resilience to control conditions comparatively to single species biofilms.

RESUMO

O fornecimento de água potável (AP) é um assunto de prioridade máxima de qualquer sociedade civilizada. A AP é uma necessidade básica para o desenvolvimento humano, saúde e bem-estar. O principal desafio da indústria da água é fornecer um produto que é microbiologicamente e quimicamente seguro, esteticamente agradável e em quantidade e pressão adequadas. Normalmente, a água à saída da estação de tratamento tem qualidade, mas esta diminui ao longo do seu percurso no sistema de distribuição de água potável (SDAP). É sabido que os biofilmes constituem um dos principais problemas microbianos dos SDAP, que mais contribuem para a deterioração da qualidade da água. Contudo, a sua eliminação destes sistemas é quase impossível, mas vários aspectos podem ser manipulados de modo a prevenir e controlar o seu crescimento.

O objectivo principal deste trabalho é contribuir para melhor entender os mecanismos, biológicos e ecológicos (adesão, coagregação, diversidade e interacções microbianas, resistência a antimicrobianos), envolvidos na formação de biofilme em SDAP, com o intuito de controlar e prevenir a sua formação, de modo a melhorar a qualidade da AP. Assim, vários aspectos foram estudados nomeadamente: a formação de biofilme por bactérias autóctones da AP, usando biorreactores (efeito das condições hidrodinâmicas e material suporte); isolamento e identificação das bactérias autóctones presentes na AP local; estudos da sua capacidade de adesão e formação de biofilme (efeito das propriedades físico-químicas da superfície das bactérias e materiais); estudos da capacidade de coagregação e interacções bacterianas inter-espécie envolvidas na formação de biofilme, detecção de metabolitos microbianos secundários com propriedades antimicrobianas e com potencialidade no controlo de biofilmes; estudo do controlo de biofilmes simples e multiespécie com hipoclorito de sódio (SHC) e metabolitos microbianos.

O estudo da formação de biofilme foi efectuado em dois biorreactores diferentes, células de fluxo e Propella^{TM}, de modo a estudar a influência das condições hidrodinâmicas (escoamento turbulento e laminar) e materiais (PVC e SS) na formação de biofilme. Os biofilmes foram monitorizados ao longo do tempo em termos de bactérias totais e cultiváveis. Todas as condições permitiram a formação de biofilme. A formação de biofilme em escoamento turbulento foi semelhante nos dois biorreactores, independentemente do material. Em escoamento laminar, no Propella^{TM} obteve-se maior formação de biofilme que nas células de fluxo. A formação de biofilme nas células de fluxo foi maior em PVC do que em SS. Enquanto, no Propella^{TM} a formação de biofilme foi semelhante em PVC e SS para ambos os regimes de escoamento. Este trabalho também possibilitou o isolamento de diferentes bactérias da AP, as quais foram identificadas; algumas delas foram seleccionadas para os estudos posteriores.

As bactérias da AP seleccionadas (25 fenotipicamente diferentes) e alguns materiais frequentemente usados nos SDAP (SS304, SS316, cobre, PP, PE, silicone e vidro) foram caracterizados de acordo com as propriedades superficiais (tensão superficial e hidrofobicidade) pela medição dos ângulos de contacto e o seu potencial de adesão foi estudado pela teoria termodinâmica e por ensaios de adesão em microplacas. Todas as bactérias foram classificadas como hidrofílicas e os materiais como hifrofóbicos. SS304, cobre, PP, PE e silicone favorecem termodinamicamente a adesão para a maioria das estirpes testadas, enquanto a adesão foi geralmente termodinamicamente menos favorável para o SS316, PVC e vidro. Os ensaios de adesão mostraram que para além das propriedades superficiais, as características e mecanismos biológicos podem estar envolvidos nos processos iniciais de adesão e podem ter um papel determinante na capacidade de adesão. Consequentemente, o estudo da adesão unicamente baseado na teoria termodinâmica não fornece resultados precisos e fiáveis. Este trabalho também sugere que bactérias com elevada capacidade de adesão (*A. calcoaceticus*) podem ter um papel decisivo na colonização primária de superfícies e possivelmente na formação inicial de biofilme em ambientes reais.

Os estudos da capacidade de adesão e formação de biofilme por seis bactérias da AP seleccionadas (*A. calcoaceticus, B. cepacia*, *Methylobacterium* sp., *M. mucogenicum, Sph. capsulata* and *Staphylococcus* sp.) foram realizados para caracterizar as relações entre os dois fenómenos. A adesão foi avaliada pelos métodos acima referidos e a formação de biofilme foi efectuada ao longo do tempo em microplacas. A capacidade de adesão e formação de biofilme encontram-se apenas correlacionadas nas fases iniciais de formação de biofilme (24 h). Para

períodos mais longos (48 e 72 h) algumas bactérias classificadas como não aderentes produziram grandes quantidades de biofilme maduro. A adesão inicial não prevê a capacidade das bactérias da AP formarem biofilmes maduros, sugerindo que outros eventos tais como alteração fenotípica e genética durante a formação de biofilme e a produção de EPS possam ter um papel importante na formação e diferenciação do biofilme. *A. calcoaceticus*, *Methylobacterium* sp. e *M. mucogenicum* foram as bactérias com maior capacidade de produção de biofilme.

A capacidade de coagregação intergenérica entre as bactérias foi estudada (método visual e microscópico) bem como as moléculas da superfície (açúcares e proteínas) envolvidas nesse processo. Além disso, foi avaliado o papel de bactérias de ligação na formação de biofilmes multiespécie em microplacas. Os resultados demonstraram que apenas *A. calcoaceticus* autoagregou e coagregou com quatro das cinco bactérias testadas (excepção é *Methylobacterium* sp.). Este mecanismo de adesão celular é mediado por interacções lectinas-açúcares. *A. calcoaceticus* demonstrou uma possível função de ligação nos biofilmes multiespécie, sendo a sua presença nos biofilmes uma vantagem na colonização de superfícies.

A formação de biofilmes simples e duplos pelas bactérias da AP, e respectivas actividades específicas, foram efectuados em microplacas ao longo do tempo, de modo a estudar o papel das interacções bacterianas na formação de biofilme. Uma série de estudos planctónicos (avaliação da taxa de crescimento, mobilidade e produção de moléculas inibidoras do quorum sensing - QSI) também foram realizados com o intuito de identificar atributos essenciais que regulam as interacções microbianas. Evidências de sinergia/cooperação, antagonismo e interacções neutras foram identificadas entre as bactérias. *B. cepacia* tem a maior taxa de crescimento e mobilidade, e produz QSI. *Methylobacterium* sp., *Sph. capsulata* e *Staphylococcus* sp. também produzem QSI. A formação de biofilmes duplos parece apenas ser regulada pelos QSI produzidos por *Sph. capsulata* e *Staphylococcus* sp. e a elevada taxa de crescimento de *Sph. capsulata* para os seguintes biofilmes: *Sph. capsulata-M. mucogenicum*, *Sph. capsulata-A. calcoaceticus* e *M. mucogenicum-Staphylococcus* sp.

O controlo químico de biofilmes simples e multiespécie com SHC foi estudado em microplacas. A capacidade dos biofilmes recuperarem da desinfecção também foi avaliada. Os biofilmes simples de *Methylobacterium* sp. e *M. mucogenicum* tiveram a maior resistência a SHC, enquanto *Staphylococcus* sp. e *A. calcoaceticus* formam os biofilmes mais susceptíveis. Em geral, os biofilmes multiespécie foram mais resistentes à inactivação e remoção do que os simples. Os biofilmes multiespécie com as seis bactérias tiveram a maior resistência a SHC, enquanto os biofilmes sem *A. calcoaceticus* foram os mais susceptíveis. Apenas os biofilmes sem *A. calcoaceticus* não foram capazes de recuperar a sua biomassa após o tratamento com SHC. *A. calcoaceticus* tem um papel fundamental na resistência e resiliência dos biofilmes da AP (a sua presença nos biofilmes multiespécie aumenta a sua resistência e a sua capacidade de recuperação).

Os efeitos dos metabolitos extracelulares na formação de biofilmes simples e multiespécie foram estudados em microplacas. Além disso, também foram caracterizados aspectos fisiológicos adicionais das bactérias que regulam as interacções inter-espécie (taxas de crescimento planctónico e séssil, actividade antimicrobiana, produção de QSI, moléculas QS, e chelantes de ferro) com o intuito de identificar espécies bacterianas com potencial biocontrolo. Os biofilmes simples de *A. calcoaceticus*, *B. cepacia*, *Methylobacterium* sp. e *M. mucogenicum* foram fortemente inibidos pelos sobrenadantes das outras bactérias. Os sobrenadantes da *M. mucogenicum e Sph. capsulata* demonstraram um elevado potencial para inibir o crescimento dos biofilmes das outras bactérias. Para os biofilmes multiespécie apenas os sobrenadantes produzidos por *B. cepacia* e *A. calcoaceticus* não tiveram efeitos inibitórios (causaram potenciação) na formação de biofilme. Os biofilmes multiespécie foram altamente susceptíveis aos metabolitos na ausência da *A. calcoaceticus*.

Em conclusão, o trabalho desenvolvido nesta tese demonstra que *A. calcoaceticus* tem uma elevada capacidade de adesão e coagregação e apresenta resistência e resiliência a condições de controlo. A presença desta bactéria no consórcio estudado representa uma vantagem significativa de colonização. Parece ser estratégico reflectir sobre a presença desta bactéria no sistema local de AP como uma previsão da presença de biofilmes resistentes ao cloro. Além disso, a associação de diversas espécies microbianas e as interacções inter-espécie aumentam a resistência e resiliência dos biofilmes multiespécie comparativamente aos biofilmes simples.

THESIS OUTPUTS

Part of the results presented in this thesis has been published/submitted elsewhere.

Papers in international scientific periodicals with referees:

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Abbreviations

AHL – *N*-acyl-homoserine lactones

AI – Auto-inducer

ASI – American steel institute

ATP – Adenosine triphosphate

CAS – Chrome azurol S

CFU – Colony forming units

CV – Crystal violet

2D – Two-dimensional

DAPI – 4,6-diamino-2-phenylindole

DLVO – Derjaguin-Landau-Verwey-Overbeek

DNA – Deoxyribonucleic acid

DW – Drinking water

DWDS – Drinking water distribution system

EPS – Extracellular polymeric substances

GAC – Granular activated carbon

HACCP – Hazard analysis critical control points

HIV – Human immunodeficiency virus

HPC – Heterotrophic plate counts

IWMI – International water management institute

L/D – Live/Dead® *Bac*Light Bacterial viability kit

mDPE – Medium density polyethylene

OD – Optical density

PBS – Phosfate buffer saline

PCR – Polymerase chain reaction

PE – Polyethylene

PI – Propidium iodide

PMS – Phenazine methosulphate

PP – Polypropylene

PS – Polystyrene

PVC – Polyvinyl chloride

QS – Quorum sensing

QSI – Quorum sensing inhibitors

R2A – R2A nutrient agar medium

rRNA – Ribossomal ribonucleic acid

rpm – Rotations *per* minute

SD – Standard deviation

SEM – Scanning electron microscopy

SHC – Sodium hypochlorite

SPSS – Statistical package for the social siences

SS – Stainless steel

TB – Total bacterial cell counts

TBX – Tryptone bile X-glucuronide

TSA – Trypticase soy agar

TSB – Trypticase soy broth

UNDP – United Nations development programme

uPVC – Unplasticized polyvinyl chloride

UV – Ultraviolet

VBNC – Viable but non-cultivable cells

WHO – World health organization

XTT – Benzene sulfonic acid hydrate

Symbols

P – Statistical significance level

v – Volume

w – Weight

θ_i – Contact angle [i = B (α – bromonaphtalene); i = F (formamide); i = W (water)] (º)

γ_i^- – Electron donor surface tension parameter [i = b (bacteria); i = s (support material)] (mJ m^{-2})

γ_i^+ – Electron acceptor surface tension parameter [i = b (bacteria); i = s (support material)] (mJ m^{-2})

γ_i^{AB} – Polar (Lewis acid-base) surface tension parameter [i = b (bacteria); i = s (support material)] (mJ m^{-2})

γ_i^{LW} – Apolar (Lifshitz-van der Waals) surface tension parameter [i = b (bacteria); i = s (support material)] (mJ m^{-2})

γ_i^{TOT} – Total surface free energy tension [i = b (bacteria); i = s (support material)] (mJ m^{-2})

ΔG_{iwi}^{TOT} – Hydrophobicity expressed as the total free energy of interaction between two entities of that material (i) immersed in water (w) [i = b (bacteria); i = s (support material)] (mJ m^{-2})

ΔG_{bws}^{TOT} – Total free energy of adhesion between the bacteria (b) and the support material (s) when immersed in water (w) (mJ m^{-2})

CHAPTER 1 THESIS OVERVIEW

This chapter provides the context and motivation, the aims and general framing of this thesis, working as a guide line to the overall works presented in the further chapters.

1.1 Context and motivation

The main goal of water companies is to deliver to each consumer microbiological safe drinking water (DW), adequate in quantity and delivery pressure and acceptable in terms of taste, odour and appearance. Studies in a full-scale DW distribution system (DWDS) indicated that most bacteria derived from the biofilm of pipeline surfaces. DWDS are known to harbour biofilms, even if in the presence of a disinfectant. Biofilms are constituted by a microbial community adapted to conditions of low nutrient concentration and high chlorine levels. The presence of biofilms in DWDS constitutes one of the currently recognized hazards affecting the microbiological quality of DW and may lead to a number of unwanted effects on the quality of the distributed water. Microbial growth may affect the turbidity, taste, odour and colour of the water, contribute to the increase of the amount of cells in the bulk phase, promote the deterioration of metallic pipes, induce a disinfectant demand and therefore promote disinfectant decay in distribution system. Also, biofilms can constitute a reservoir of pathogenic microorganisms.

The development of biofilms in DWDS is influenced by several factors, including microbial quality of intake water, concentration of biodegradable organic matter, amount of available nutrients, sediment accumulation, concentration of residual disinfectants, water residence time, environmental factors (pH, temperature and turbidity of the water), design of network (presence of dead ends, diameter of pipes), hydrodynamics (shear stress at the biofilm-liquid interface), characteristics of material covering the distribution pipes (composition, porosity, roughness) and their conservation state. Recent studies into the microbial ecology and population dynamics of DWDS have found that other important mechanisms play a determinant role in DWDS biofilm formation and on their resistance to disinfectants. Those include the microbial diversity, interspecies interactions, autoaggregation and coaggregation, presence/release of microbial metabolites and molecules (cell-cell signalling), and transfer of genetic material. However, the role of those mechanisms in DWDS biofilm formation remains poorly understood. The purpose of the work developed in this thesis is to gain deeper insights into the biological and ecological mechanisms relevant for biofilm formation in DWDS. The results obtained with this work

can contribute for the development of novel and more effective biofilm control practices in DWDS.

1.2 Aims and outlines

The main goal of the investigation presented in this thesis is to provide a contribution to better understand the fundamental biological and ecological mechanisms involved in biofilm formation in DWDS, with the intent to control and prevent their formation, in order to improve DW quality. Therefore, for the accomplishment of this main aim a research strategy was followed: isolation and identification of the autochthonous bacterial population with the ability to form biofilms using bioreactors, and were present in local DW; characterization of bacteria and support materials in terms of surface properties; studies on their adhesion, characterization of coaggregation and biofilm formation abilities; assessment of interspecies interactions involved in biofilm formation; detection of microbial secondary metabolites with antimicrobial and biofilm control properties; study on single and multispecies biofilm control with sodium hypochlorite (SHC) and microbial metabolites.

This thesis is divided in ten chapters:

Chapter 1 shows the context and motivation of this work, as well the main objectives.

Chapter 2 encloses the literature review, where it is reviewed current scientific knowledge on biofilm formation and control in DWDS and its relevance in DW quality and public health. Briefly, this chapter describes the characteristics of DW industry - from nature to tap; the main DW quality problems and its implication on health; the biofilm formation and the main factors that affect its accumulation in DWDS; the mechanisms of biofilm formation in DWDS; and the strategies to control biofilms in DWDS. The next seven chapters correspond to different parts of the experimental work developed in this thesis.

Chapter 3 describes the strategy used to monitor biofilm formation by DW autochthonous bacteria under different process conditions using two distinct bioreactors, Propella^{TM} and flow cell, in order to study the influence of hydrodynamic conditions and support materials on biofilm formation. This work also allowed the isolation of

heterotrophic bacteria colonizing the biofilms. Those bacteria were used to study relevant aspects promoting biofilm formation whose results are presented in the subsequent chapters. In chapter 4, 25 phenotypically distinct bacteria belonging to 14 different bacterial species were used. Nevertheless, for the work presented in the following chapters were only selected 6 representative DW-isolated bacteria, recognized as problematic opportunistic bacteria with the potential to cause public health problems.

Chapter 4 shows the characterization of 25 DW-isolated heterotrophic bacteria and some support materials commonly used in DW networks, in terms of physicochemical surface properties (surface tensions and hydrophobicity) by means of contact angles measurements, and the study of adhesion potential. The adherence characteristics of DW bacteria to materials were determined by two methods: thermodynamic prediction of adhesion (free energy of adhesion) and by adhesion assays using microtiter plates.

Chapter 5 provides the study of adhesion and biofilm formation by DW bacteria. The understanding of the overall biofilm process depends on the deep understanding of the main aspects regulating the biofilm development, such as the initial adhesion. This study was performed in order to characterize the relationships between adhesion and biofilm formation abilities of DW-isolated bacteria. Adhesion was assessed by two distinct methods: thermodynamic and microtiter plate assay. The biofilms were developed in microtiter plates and analyzed over time. The understanding of this relationship is important for the development of control strategies in the early stages of biofilm formation.

Chapter 6 concerns the study of intergeneric coaggregation between DW bacteria. Coagregation was studied by a visual assay and by two microscopic techniques (epifluorescence and scanning electron microscopies). Extracellular proteins and polysaccharides were assessed over time and correlated with coaggregation ability. The surface-associated molecules involved in coaggregation process were investigated by heat and protease treatment, and by sugar reversal tests. The role of *Acinetobacter calcoaceticus* as a bridging organism in DW biofilms was assessed by multispecies biofilm experiments in microtiter plates, through strain exclusion tests.

In **Chapter 7** it is presented the study of bacterial interactions in DW biofilms. The aim of this work was to assess the role of interspecies interactions in dual species biofilm formation and characteristics. Single and dual species biofilm formation, and specific activities, by DW-isolated bacteria were determined using microtiter plates over a 72 h period. A series of planktonic studies were also performed, assessing the bacterial growth

rate, motility and production of quorum sensing inhibitors (QSI), in order to try to identify key attributes regulating microbial interactions between DW bacteria.

Chapter 8 regards the study of chemical disinfection to control DW biofilms. The knowledge of the role of microbial diversity of DW biofilms on disinfection might help to improve the understanding of their resistance mechanisms and allow the development of effective strategies to apply in DWDS. In this work the effects of SHC on the control of single and multispecies biofilms formed by DW-isolated bacteria in microtiter plates were studied. Furthermore, the biofilm ability to recover from disinfection was assessed.

Chapter 9 displays the study on the use of bacterial metabolites to control DW biofilms. The aim of this study is to understand the effects of metabolite molecules produced by DW-isolated bacteria on biofilm formation and development and to evaluate their potential as biocontrol strategy. Single and multispecies biofilms in the presence and absence of cell-free supernatants produced by partner bacteria were performed in microtiter plates. Moreover, bacterial physiological aspects regulating interspecies interactions, such as planktonic and sessile growth rates, cell-free supernatant antimicrobial activity, and production of QSI, quorum sensing (QS) molecules and iron chelators were characterized with the intent to identify bacterial species with biocontrol potential.

Chapter 10 gives an overview of the work done in this thesis, the main conclusions obtained, and some suggestions for further research are also presented.

CHAPTER 2 LITERATURE REVIEW

This chapter reviews current scientific knowledge on biofilm formation and control in drinking water distribution systems and its relevance in drinking water quality and public health.

Parts of the contents presented in this chapter were published on:

Simões LC, Simões M and Vieira MJ **(2007)** The role of microbial interactions in drinking water biofilms. *In:* Biofilms: coming of age. Gilbert P, Allison D, Brading M, Pratten J, Spratt D and Upton M (eds.). The Biofilm Club, Cardiff, Wales, pp. 43-52.

Simões M**,** Cleto S, **Simões LC,** Pereira MO and Vieira MJ **(2007)** Microbial interaction in biofilms – the role of siderophores and iron-dependent mechanisms as biocontrol strategies. *In:* Biofilms: coming of age. Gilbert P, Allison D, Brading M, Pratten J, Spratt D and Upton M (eds.). The Biofilm Club, Cardiff, Wales, pp. 157-165.

Simões M, **Simões LC** and Vieira MJ **(2010)** A review of current and emergent biofilm control strategies. ***LWT – Food Science and Technology*** **43**: 573-583.

2.1 Drinking water

Water is the most common and important chemical compound on earth. It is essential for all socio-economic development and for maintaining healthy ecosystems. Only approximately 2.6% of the global water amount of 1.4×10^9 km^3, is freshwater and consequently available as potential DW. The availability of safe DW has been the most critical factor for survival during the development of all life (Szewzyk *et al.*, 2000). DW or potable water is a product of sufficiently high quality that can be consumed or used without risk of immediate or long term harm. In most developed countries, the water supplied to households, commerce and industry is all of DW standard, even though only a very small proportion is actually consumed or used in food preparation.

2.1.1 Brief history

In the history of humankind, cultural centers were always founded in areas with a sufficient amount of freshwater. The problems with water arguably began to emerge when man became sessile and established permanent settlements. The formation of towns and cities resulted in an increase in demand for freshwater and first efforts to supply these cities with clean water by means of channels, aqueducts and pipes, as well as, to obtain access to new water reservoirs (drilling of wells). Early records of the use of water distribution systems date back to 2000 - 1500 BC (Needham and Ling, 1965; Deming, 1975) and are summarized in Table 2.1.

Initially, developing communities found that supplying and distributing a sufficient volume of DW presented major problems. But very soon other complications of highly populated areas emerged, such as increasing amounts of waste, wastewater, and other types of contamination, also endangered access to fresh and safe DW.

Besides hygienic problems caused by unsanitary waste, the rapid development of industry, especially the development of the chemical industry, has resulted in an ever present contamination of all kinds of natural water systems.

Table 2.1 List of ancient water supply systems (adapted from Bachmann and Edyvean, 2005)

Time	Method of water supply	Origin of water	Primary culture / geographical locations	Potential source of contamination	Water treatment	Use
Approx. 10000 BC (before cities)	Bucket system	Spring water, rainwater, lake	Gathering	Atmosphere (dust, acid rain), lithosphere (dissolution of minerals), biosphere (e.g. faeces from wildlife and humans)	Unknown	Drinking, cooking
Approx. 2000 BC	Hollowed wood pipes and copper tubing	Surface water	Agriculture / Egypt	Human activity (agriculture manufacturing, mining), biosphere	Unknown	Irrigation, bathing
Approx. 1500 BC	Canals and reservoirs	River	Agriculture / Egypt, Babylonia, Assyria, China, Middle East	Atmosphere, lithosphere, biosphere, human activity	Sedimentation and wick siphons	Drinking, brewing, baking, irrigation
Approx. 700 BC	Qanats (subterranean aqueducts)	Rain water	Agriculture / Persia, Turpan (China)	Lithosphere, biosphere, human activity	Gravity-driven filtration	Irrigation, drinking
Approx. 700 BC	Aqueducts	River, lake	Agriculture / Jerusalem (Israel), Samos (Greece)	Atmosphere, biosphere, human activity	Sedimentation (cistern)	Drinking, bathing, cooking, irrigation
Approx. 300 BC	Pentagonal stoneware piping	Surface water	Agriculture / Wei valley (China)	Biosphere, human activity	Unknown	Drinking, cooking
Approx. 300 BC	Lead pipes	River, lake	Agriculture / Greece, Italy	Biosphere, human activity, cistern	Sedimentation (cistern)	Drinking, bathing, cooking
Approx. 180 BC	Bronze pipes	Surface water	Agriculture / Pergamon	Biosphere, human activity	Unknown	Drinking, bathing, cooking
Approx. 206 BC to AD	Bamboo and terracotta pipes	Surface water	Agriculture / China	Biosphere, human activity	Unknown	Drinking, bathing, cooking, irrigation, mining

A vague understanding of the need to protect water systems that are used for DW from contamination with waste and wastewater is documented in historic documents, for instance in the Bible. The necessity of resource protection and DW treatment became evident when the connection between bacteria in DW and the outbreak of various diseases was made. One of the first outbreaks from which this was evidenced was the 1919 typhoid fever outbreak in Pforzheim (Germany), which caused 4000 cases of diseases and resulted in 400 deaths. In this epidemic, it was possible to prove that the DW was contaminated by sanitary waste that was applied as fertilizer (Szewzyk *et al.*, 2000).

Water quality in ancient times was assessed by its effects on human health and according to organoleptic characteristics, namely colour, taste and smell (Bachmann and Edyvean, 2005). In the late 19th century, water professionals and consumers throughout the

world still use tastes and odours to assess water quality. Advances in microbiology, the use of microscopes and increased public health concerns in the early 20th century, caused sanitation and disinfection to become drivers for water quality. In the mid and late 20th century, scientific advances in chemistry and analytical instrumentation enabled monitoring of inorganic and organic chemicals in DW. Upon discovering that some of these chemicals were toxicants, regulations of chemical species became another factor for water quality. In the late 20th century, the DW industry rediscovered aesthetics and began to adapt sensory assessment methods from the food and beverage industry so that a microbial safe, chemical safe and palatable product could be delivered to consumers (Cairncross and Sjóström, 1950; Krasner *et al.*, 1985; Bruvold, 1989; APHA, 1995; Dietrich *et al.*, 2003; Dietrich, 2006). At the beginning of the 21st century, all three of these factors – microbiology, chemicals, and aesthetics – are foci for consumers, water producers, and regulatory agencies related to the delivery of safe DW (Dietrich, 2006).

Nowadays, in most industrialized countries, DW is ranked as food, and high standards are set for its quality and safety. The strict requirements for microbiological factors specify that bacterial content should be very low and that no pathogenic or potentially pathogenic microorganisms should be detectable. Therefore, with the publication of DW standards such as the European Union DW Council Directive 98/83/EC, water must conform to the standards laid down for a large number of diverse parameters (microbial, chemical and physical). According to this legislation and World Health Organization (WHO) guidelines, DW should contain pathogenic microorganisms only in such low numbers that the risk for acquiring waterborne infections is below an acceptable limit. The fulfilment of these requirements demands resource protection and careful treatment of raw water, as well as accurate quality control of the treatment process and distribution, should allow supply safe DW to consumers.

2.1.2 Drinking water industry: from nature to tap

The main challenge to the DW industry is to produce water that is microbiologically and chemically safe, plus aesthetically pleasing. Therefore, professionals in all fields, physical sciences, biological sciences, social sciences, engineering, and medicine, are necessary to overcome this challenge.

The water supply industry is vitally important not only to maintain the health of the community, but also for the sustainability of industry, business and agriculture. The human dependence on treated water is now incalculable, and threats to that supply are comparable to the worst natural and man-made disasters. The volumes of water consumed each day by agriculture, industry and the public are vast, requiring an enormous infrastructure to satisfy the demand. Like the other service providers, electricity, telephone and gas, the water utilities deliver their product to the home, which requires a network of distribution pipes to service each household, but unlike the other utilities these are stand alone local or regional networks, rather than integrated national supply networks.

2.1.2.1 Water resources: availability and scarcity

Water resources are sources of water that are useful or potentially useful to humans. Uses of water include agricultural, industrial, household, recreational and environmental activities. Virtually, all of these human uses require freshwater. However, the amount of freshwater on earth is limited, and its quality is under constant pressure. Water is constantly being recycled in a system known as the hydrological cycle.

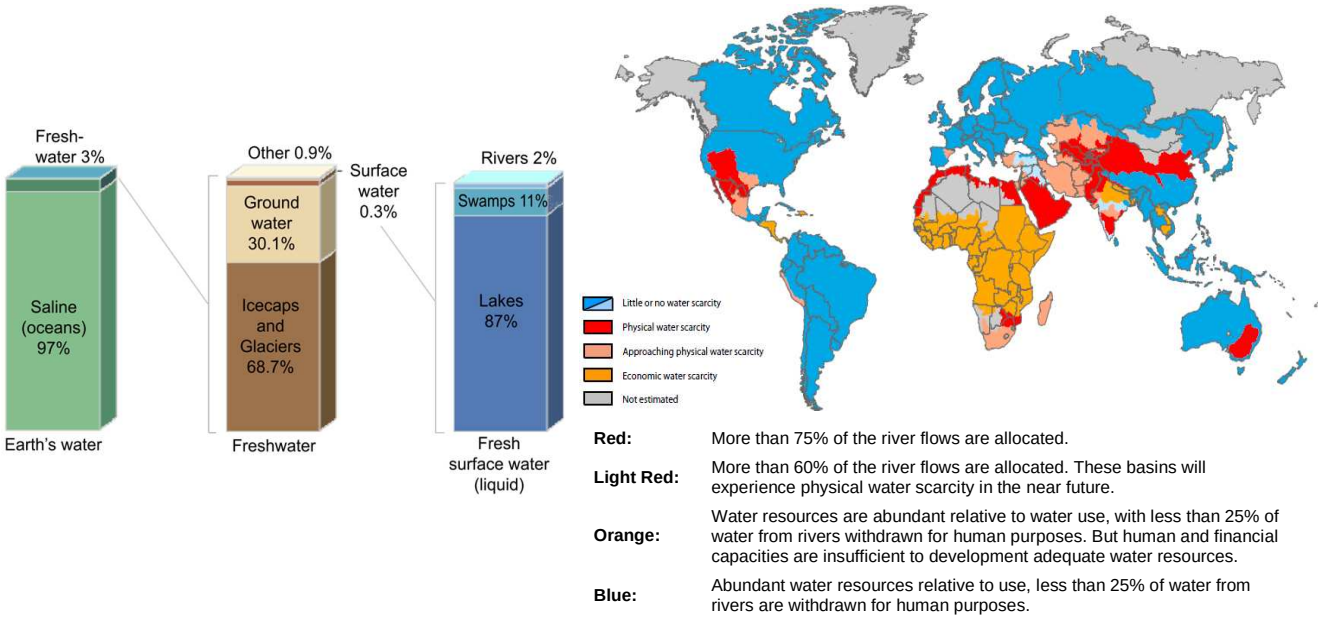


Figure 2.1 http://ga.water.usgs.gov/edu/waterdistribution.html); (b) Areas of water scarcity in the world (source: IWMI report, 2006).

While the total volume of water in earth remains constant, its quality and availability varies significantly. In terms of total volume, about 97% of the earth's water is saline and is

in the oceans. This means that only 3% of the volume of water in the world is actually non-saline (Figure 2.1a). However, not all of this freshwater is readily available for use by humans. About 68.7% is currently locked up as ice caps and glaciers, with a further 30.1% located underground as groundwater, which means that approximately 1% of the total fresh water is found in lakes, rivers and the soil. The water present in soil moisture represent a small percentage but unavailable to humans for supply. Therefore, only 0.3% of the earth's water budget is present in lakes, swamps and rivers. It represents the fresh surface water available to human for supply (Figure 2.1a). Water supplies, therefore, come from two principal resources within the water cycle: surface and groundwater.

Water scarcity is both a natural and a human-made phenomenon. There is enough freshwater on the planet for six billion people but it is distributed unevenly and too much of it is wasted, polluted and unsustainably managed. Water scarcity is among the main problems to be faced by many societies and the world in the 21st century (UNDP, 2006).

Despite freshwater to be a renewable resource, yet the world's supply of clean water is steadily decreasing. Water demand already exceeds supply in many parts of the world. Water shortages is influenced by the continue growth of population, the expansion of business activity, urbanization, climate changes, depletion of aquifers and the degradation of groundwater and surface water quality. Water quality degradation can be a major cause of water scarcity in both developed and developing countries. The water scarcity is the imbalances between availability and demand, the degradation of surface and groundwater quality, intersectoral competition, interregional and international conflicts. Water conflicts can arise in water stressed areas among local communities and between countries because sharing a very limited and essential resource is extremely difficult.

Water use has been growing at more than twice the rate of population increase in the last century, and, although there is no water scarcity, an increasing number of regions are short of water. Water scarcity already affects every continent (Figure 2.1b). Around 1.2 billion people, almost one-fifth of the world's population, live in areas of physical scarcity, and 500 million people are approaching this situation. Another 1.6 billion people, or almost one quarter of the world's population, face economic water shortage (where countries lack the necessary infrastructure to take water from rivers and aquifers). It has been estimated that, by 2025, 1800 million people will be living in countries or regions with absolute water scarcity, and two-thirds of the world population could be under stress conditions. Economic scarcity occurs when there is a lack of investments in water or lack of human capacity to

keep up with growing water demand. Symptoms of economic water scarcity include deficient infrastructure development where the people have troubles getting enough water for human purposes; or inequitable distribution of water even though infrastructure exists. On the other hand, physical scarcity occurs when available resources are insufficient to meet all demands. Arid regions are most often associated with physical water scarcity. Pressure on water resources is particularly acute in arid regions that support agricultural production or large populations, regions where water use is high relative to water availability. The Middle East, Central Asia, North Africa, South Asia, China, Australia, the western United States, and Mexico are especially prone to water shortages. However, an increasing number of countries can be considered water-stressed (UN-Water, 2006).

2.1.2.2 Sources of drinking water

The origin of drinking water, as already said, can be superficial (including streams, rivers, lakes and dams) or groundwater (such as wells, springs and holes). A number of factors have entered into the choice of a best available raw source of water for potable supply. These include adequate quantity during any seasonal variations in flow, water quality that is amenable to cost-effective treatment and some measure of watershed protection from domestic, industrial and agricultural pollution (Geldreich, 1996).

The quality and quantity of surface water depends on a combination of climatic and geological factors. The quality of river water is also an important factor. River water requires complex and expensive treatment before being supplied to the consumer. The complexity and cost of treatment increases as the quality of the raw water deteriorates. Economically groundwater is much cheaper than surface water, as it is available at the point of demand at relatively little cost and it does not require the construction of reservoirs or long pipelines. It is usually of good quality, usually free from suspended solids and, free from bacteria and other pathogens, except in limited areas where it has been affected by pollution. Therefore, it does not require extensive treatment before use.

2.1.2.3 Water consumption

Water demand varies significantly between countries due to differences in culture, climate and economic wealth (Smith and Ali, 2006). Figure 2.2 compares the average daily volume of water used per capita in a variety of countries with people in the USA and Australia using up to 40 to 60 times more than people in some water scarce areas. The

minimum requirement for water has been estimated as 50 l per capita per day, the so-called water poverty level. This includes 5 l for drinking, 20 l for sanitation, 15 l for bathing and 10 l for food preparation. The amount of water a person has access to, should in theory be based on the amount of water potentially available. It is generally accepted by hydrologists that the threshold between a country having adequate water resources, and not, is 1700 $m^3ca^{-1}yr^{-1}$ i.e. the water stress threshold. In practice very few countries use that much water. For example, in Europe the average per capita usage is 726 $m^3ca^{-1}yr^{-1}$, although in North America it is double (1693 $m^3ca^{-1}yr^{-1}$). Each one of us uses on average between 150 and 580 l each day, with the UK having one of the lowest per capita water consumption rates in the developed world, at 150 l (Figure 2.2). Nevertheless, only less than 5% of this will be consumed or used for cooking. With leakage rates as high as 40% in some distribution systems, it seems strange that we have to treat all this water when so little needs to be of the very best quality (Gray, 2008)

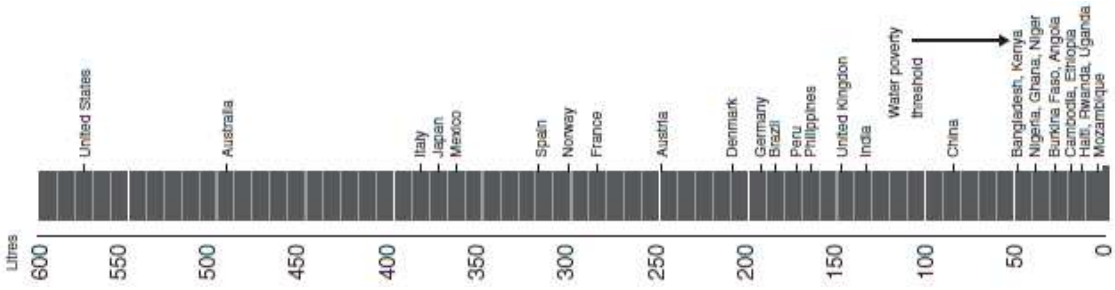


Figure 2.2 Comparison of the average volume of water used per person per day in 2002, and those below the water poverty threshold of 50 l ca^{-1} d^{-1} (adapted from Gray, 2008).

The demand for water also varies over the 24 hour period. This is known as the diurnal variation, with peak usage occurring between 7 and 12 h and from 18 to 20 h each day. Demand is higher during weekends by about 12%, with demand being higher in the summer than in the winter (Gray, 2008).

2.1.2.4 Water treatment and distribution

Water treatment and distribution is the process by which water is taken from water resources, made suitable for use and then transported to the consumer. This is the first half of the human or urban water cycle, before water is actually used by the consumer. The second half of the cycle is the collection, treatment and disposal of used water (sewage) (Gray, 2004). These processes are represented in a schematic diagram (Figure 2.3) showing the role of the water companies in supplying water to the consumer and subsequently

treating it before returning it to the hydrological cycle (Latham, 1990). However, this literature review only does reference to the processes of DW production and supply which occur in the first half of the urban water cycle.

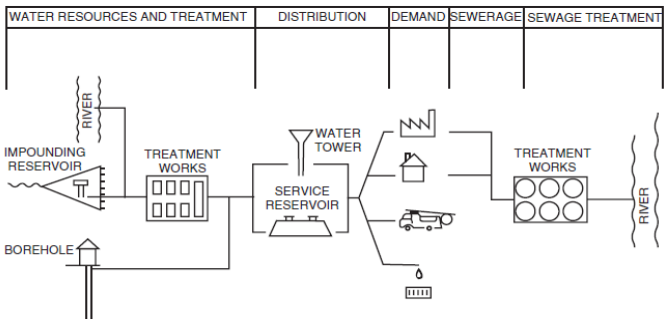


Figure 2.3 Schematic diagram of the human or urban water cycle. Demands shown are industrial, domestic, fire-fighting and leakage (adapted from Gray, 2008).

Water is a substance which rapidly absorbs both natural and man-made substances, generally making the water unsuitable for drinking without some form of treatment. Important categories of substances that can be considered undesirable when in excess are indicated in Table 2.2.

Table 2.2 Parameters of water quality and their meaning (adapted from Gray, 2008)

Parameters of quality	Meaning
Colour	Due to the presence of dissolved organic matter from peaty soils, or the mineral salts of iron and manganese
Suspended matter	This is fine mineral and plant material that is unable to settle out of solution under the prevailing conditions
Turbidity	A measure of the clarity, or transparency, of the water. Cloudiness can be caused by numerous factors such as, fine mineral particles in suspension, high bacteria concentrations, or even fine bubbles due to over-aeration of the water
Pathogens	These can be viruses, bacteria, protozoa or other types of pathogenic organism that can adversely affect the health of the consumer. They can arise from animal or human wastes contaminating the water resource
Hardness	Excessive and extremely low hardness are equally undesirable. Excessive hardness arises mainly from groundwater resources whereas very soft waters are characteristic of some upland catchments
Taste and odour	Unpleasant tastes and odours are due to a variety of reasons such as contamination by wastewaters, excessive concentration of certain chemicals such as iron, manganese or aluminium, decaying vegetation, stagnant conditions due to a lack of oxygen in the water, or the presence of certain algae
Harmful chemials	There is a wide range of toxic and harmful organic and inorganic compounds that can occur in water resources. These are absorbed from the soil or occur due to contamination from sewage or industrial wastewaters

The objective of DW treatment is to produce an adequate and continuous supply of water that is chemically, bacteriologically and aesthetically pleasing. More specifically, water treatment must produce water that has the characteristics presented in Table 2.3.

Table 2.3 Characteristics of DW after adequate treatment

Characteristics of DW	Meaning
Palatable	Has no unpleasant taste
Safe	It should not contain any pathogenic organism or chemical that could be harmful to the consumer
Clear	Be free from suspended matter and turbidity
Colourless and odourless	Be aesthetic to drink
Reasonable soft	Allow consumers to wash clothes, dishes and themselves without excessive use of detergents or soaps
Non-corrosive	Not be corrosive to pipework or encourage leaching of metals from pipes or tanks
Low organic content	A high organic content will encourage unwanted biological growth in pipes or storage tanks, which can affect the quality of the water supplied

DW treatment plants must be able to produce a finished product of consistently high quality regardless the demand that fulfils the requirements of consumers and authorities defined by several organoleptic, microbiological and physicochemical parameters. Water treatment consists of a range of unit processes that are usually operated in series (Stevenson, 1998). The main unit processes used in general for DW treatment are listed in Table 2.4. However, it is not usual to have all these processes in only one treatment plant.

Table 2.4 Main unit processes in DW treatment in general order of use (adapted from Gray, 2008)

Treatment category	Intake	Pre-treatment	Primary treatment	Secondary treatment	Disinfection	Tertiary treatment	Fluoridation	Distribution
Unit process		Coarse screening Pumping Storage Fine screening Equalization Neutralization Aeration Chemical pre-treatment	Coagulation Flocculation Sedimentation	Rapid sand filtration Slow sand filtration		Adsorption Activated carbon Fe and Mn removal Membrane processes		

The selection of unit processes depends on the quality of the raw water entering the treatment plant and the quality of the finished water required. As better the quality of raw water, lesser the number of unit processes needed and lesser are the overall costs of DW production. Groundwater is generally much cleaner than surface waters and so, does not require the same degree of treatment. The water industry tries to obtain the cleanest water possible for supply, although the volume and consistency of supply are the major factors in the selection of a resource.

A brief description of the major unit processes is shown below. Detailed reviews on water treatment strategies were already published (Twort *et al.*, 1994; Vigneswaran and Visvanathan, 1995).

Preliminary screening. In this process the raw water is passed through a set of coarse screens to remove gross solids such as weeds, sticks and other large material before starting its passage in the treatment plant. This is mainly carried out to protect pipes from becoming blocked or pumps from being damaged.

Storage. Raw water is pumped from the intake to the storage reservoir where it is often stored to improve quality before treatment, as well as ensuring adequate supplies at periods of peak demand. There are a number of natural processes at work during storage that all significantly improve water quality. Namely the sedimentation of particulate matter in suspension, ultraviolet (UV) radiation (destroys harmful bacteria and some other pathogenic organisms), colour bleached by sunlight, oxidation of some organic impurities in the upper zones of the reservoir and among other natural processes. Storage can largely eliminate variations in water quality which can occur in surface waters (Gray, 2008).

Screening and microstraining. Before treatment the raw water is screened again, however, through fine screens. If considerable amounts of fine solids or algae are present, then microstraining may be used before the next stage. Microstraining produces a wash-water in which all the strained particles, including the algae, are concentrated.

Aeration. Water from groundwater resources, from the bottom of a stratified lake or reservoir, or from a polluted river, will contain very little or no dissolved oxygen. If anaerobic water is allowed to pass through the treatment plant it will damage or affect other unit processes, in particular filtration and coagulation. Therefore, the raw water needs to be aerated before further treatment. This is achieved by bringing the water into contact with air. There are many types of aeration systems, bubbling air, cascade or fountain system and other types including packed towers and diffusers (Twort *et al.*, 1994). Apart from ensuring

optimum treatment, aeration also provides oxygen for purification and significantly improves the quality, especially the taste, of water. Aeration also reduces certain objectionable odours, and reduces the corrosiveness of water by driving off any excess carbon dioxide gas present, thus raising the pH. Aeration cannot, however, reduce the corrosive properties of acid waters alone and neutralization with lime may be needed. Iron and manganese can also be removed from solution by aeration. These metals are only soluble in water with a pH of less than 6.5 and in the absence of dissolved oxygen, and so are common in certain groundwaters. Aeration oxidizes the soluble metal salts into insoluble metal hydroxides, which can then be removed by flocculation or filtration (Gray, 2008).

Coagulation/Floculation. After fine screening most of the remaining suspended solids will be very small, usually lesser than 10 µm. These colloidal solids are so small that they may never settle out of suspension naturally. Colloidal solids are particles of clay, metal oxides, large protein molecules and microorganisms. All small particles tend to be negatively charged, and, as like charges repel, all the negatively charged colloidal particles in the water tend to repel one another, preventing aggregation into larger particles that could then settle out of suspension. The removal of colloidal matter is a two steps process: coagulation followed by flocculation. A coagulant is added to the water to destabilize the particles and to induce them to aggregate into larger particles known as flocs. A variety of coagulants are used. The most common salts are aluminium sulphate (alum), aluminium hydroxide, polyaluminium chloride, iron (III) chloride, iron (III) sulphate and lime. The actual mechanisms of coagulation are complex and include adsorption, neutralization of charges and entrainment within the physical–chemical matrix formed (Twort *et al.*, 1994). The amount of coagulant added to the water is critical. The coagulant is added to the process stream at a specific concentration (30 - 100 mg l^{-1}) using a mixing device. Coagulation is complete within one minute of addition. Metal salts react with the alkalinity in the water to produce an insoluble metal hydroxide precipitate, which enmeshes the colloidal particles. When small particles collide in a liquid, some them naturally aggregate to form larger particles. The chance of particles colliding can be significantly increased by gently mixing the water, a process known as flocculation. When there is a high concentration of colloidal particles, then flocculation can be effective on its own. However, at the lower concentrations usually found in water resources a coagulant must be used. In the water treatment process, flocculation therefore follows chemical addition (coagulation). During

this mixing, larger flocs are produced being easily removed during clarification (Gray, 2008).

Clarification. In this process the flocs formed by the addition of a coagulant or by flocculation are removed by settlement. In water treatment the water flows in an upward direction from the base of the tank. The flocs, which are heavier than water, settle towards the bottom, so the operator must balance the rate of settling against the upward flow of water to ensure that all the particles are held within the tank as a thick sludge blanket. There is a layer of clear, clarified water at the surface that overflows a simple weir to the next step of the treatment process.

Filtration. After clarification, the water contains a small amount of fine solids (< 10 mg l^{-1}) and soluble material. Although some of these particles may have been in the natural raw water, many will have been formed during the coagulation process. Filtration is another process that is required to remove this residual material. The filters contain layers of sand (or anthracite) and gravel graded to ensure effective removal. In their simplest form, filters allow the downward passage of water through layers of fine sand, which are supported on layers of coarser gravels. Pipes at the base of the filter, underdrains, collect the filtered water. There are two types of filter used in water treatment: rapid and slow sand filters. Rapid sand filters are used for water that as previously been treated by coagulation and sedimentation, and are less effective than slow sand filters in retaining very small solids. Therefore, bacteria, taste and odours are less effectively removed than by slow sand filters. These filters apart from physical straining also provide a degree of biological treatment. Water treatment in sand filters is therefore a combination of physical and biological activity, with pathogenic bacteria, taste and odour (due to algae and organic compounds) largely removed (Gray, 2008).

pH adjustment. The pH of the finished water may require adjusting so that it is neither too acidic, which may corrode metal distribution pipes and household plumbing, or too alkaline, which will result in the deposition of salts within the distribution system causing a reduction in flow. The pH may be adjusted at a number of unit processes, such as coagulation, to ensure maximum efficiency. Alkalis such as lime, sodium carbonate or caustic soda are used to increase the pH, whereas acids are used to decrease it (Gray, 2008).

Disinfection. Although in previous processes some microorganisms have been removed, the finished water may contain pathogenic microorganisms that need to be removed or destroyed. In practice, it is impossible to sterilize water, due to the very high

concentration of chemicals required, which would make the water very unpleasant and possibly dangerous to drink. Therefore, the water is disinfected, rather than sterilized.

DW has been disinfected since the beginning of the 19th century, when discovered that microbiological contamination risks by waterborne diseases, namely cholera or typhoid fever, decreased drastically when disinfectants were used. Disinfection is used to eliminate microorganisms that have passed through the treatment processes and to ensure microbiologically safe water through the DWDS. This is achieved by adding disinfectants in excess, which will maintain a residual disinfectant during the water distribution in order to control the microbial accumulation in pipes and tanks. The used disinfection methods are chlorination, chloramination, ozonation and UV radiation (Lee and Nam, 2005). Of the all methods chlorination is by far the most widely used in DWDS.

Chlorine is a strong oxidizing agent and is the most commonly used due to its effectiveness, high solubility, stability, ease of use and low cost. Furthermore, it can provide a residual disinfectant in water that prevents or should prevent the microbial regrowth. Chlorination is normally performed with SHC that will release Cl_2 into aqueous phase by hydrolysis. Basically Cl_2 reacts with water to form hypochlorous acid (HOCl) and hydrochloric acid (HCl). In solution this reaction is very rapid and is normally complete within one second. Hypochlorous acid is a weak acid that readily dissociates into the hypochlorite ion (OCl^-), this occurs almost instantaneously. Both hypochlorous acid and the hypochlorite ion act as disinfectants, although the former is about 80 times more effective than the latter. A chemical equilibrium develops between the two forms, although dissociation is suppressed as the pH decreases. Disinfection is therefore much more effective at acidic pH. Temperature also affects chlorination, its efficiency decreasing at lower temperatures (Gray, 2008). One the main disadvantage is that free chlorine reacts with natural organic compounds and form potentially harmful by-products (Bull *et al.*, 1995; Nieuwenhuijsen *et al.*, 2000). Furthermore, chlorine may cause taste and odour problems in water. Although very effective in killing bacteria, free chlorine has a moderate effect against virus and a very limited effect against protozoa (WHO, 2006). However, there are a number of pathogenic microorganisms that are resistant to chlorine. Effectively eliminating all the coliforms present does not necessarily indicate that all other pathogenic microorganisms have also been destroyed (LeChevallier, 1990). Lisle *et al.* (1998) have demonstrated that *Escherichia coli* can survive higher concentrations than the residual chlorine concentration left in US water treatment stations. However, pathogens normally present in DW such as

Mycobacterium spp. (Le Dantec *et al.*, 2002), *Legionella pneumophila* (Kuchta *et al.*, 1983) and *Helicobacter pylori* (Baker *et al.*, 2002) were found to be more resistant to chlorination than *E. coli*, the microorganism that is routinely tested as an indicator of faecal pollution for assessing and maintaining adequate water quality. So, the role of *E. coli* has an effective indicator of treated water quality must now be questioned.

Chloramines are formed by the reaction of chlorine with the ammonia in water. These chemicals are less effective than free chlorine but are widely applied in DWDS because produces the same by-products as chlorine but in lower amounts. Their residual concentration is kept for longer periods and chloramines are not as reactive as chlorine with iron and corrosion products (LeChevallier *et al.*, 1990). However, combined residual chlorine requires a contact time of a hundred times longer than free residual chlorine to achieve the same degree of elimination of pathogens. Some reports suggest the combined use of chlorine and monochloramine in order to obtain more effective disinfection and reduce the by-products formation (Momba and Binda, 2002; Charrois and Hrudey, 2007).

Chlorine dioxide is another effective water disinfectant, but not widely used. This does not produce trihalomethanes nor react with ammonia. When applied in low amounts and concomitantly with chlorine decreased significantly the formation of trihalomethanes (Lee and Nam, 2005).

Ozone has powerful oxidation properties and has been shown to remove efficiently microorganisms, taste and odour. Ozone proved to be very effective for the inactivation of virus and protozoa that form cysts (Lee and Nam, 2005). Another advantage is that it generates relatively fewer disinfection by-products, as compared with chlorine, and leaves no taste or odour. Apart from being more expensive than chlorination, the lack of residual disinfection action in DWDS is the major drawback (Masschelein, 1982). When waters contain bromide the use of ozone should be avoided as both react and form bromate, which is widely considered to be a genotoxic carcinogen (Bull and Kopfler, 1991).

UV radiation is a non-chemical disinfection method. UV radiation is electromagnetic energy in the range 250–265 nm. This energy destroys the microorganisms by altering their genetic material, and rendering them unable to reproduce. UV radiation is a very effective disinfectant against all bacteria, virus and protozoa, cysts included (Hijnen *et al.*, 2006) in clarified waters. The main disadvantage is that UV leaves no residual disinfectant in the water, which is overcame by applying a second disinfectant to generate a residual amount.

Softening and other tertiary treatments. Conventional water treatment is unable to remove a number of soluble inorganic and non-biodegradable organic substances from water. Soluble inorganic material is removed by precipitation or ion-exchange, whereas organic substances that are not biologically degraded can be removed by adsorption using activated carbon. Membrane filtration, including reverse osmosis is also widely used for the removal of both inorganic and organic contaminants. Chemical precipitation is more widely known as precipitation softening. It is used primarily to remove or reduce the hardness in water that is caused by excessive salts of calcium and magnesium. This converts the soluble salts into insoluble ones, so that they can be removed by subsequent sedimentation. Lime or soda ash is normally used to remove the hardness Softening using ion-exchange separation is becoming increasingly common. Ion-exchange separation uses a resin, usually natural zeolites which are sodium aluminosilicates (Hill and Lorch, 1987). The zeolites exchange sodium ions for calcium and magnesium ions. The hardness is therefore removed and bound to the resin while sodium, which does not cause hardness, takes the place of calcium and magnesium in the water, making it softer. Activated carbon is used to remove trace concentrations of synthetic organic compounds, especially pesticides and industrial solvents, and other complex organic compounds responsible for taste and odour problems. Activated carbon works by adsorption of the organic molecule onto its porous structure. One of the most important advances in water treatment in the past decades has been the development of membrane filtration. Membrane filtration is widely used for the advanced and tertiary treatment of potable waters including desalination and removal of organics (reverse osmosis), softening (nanofiltration), disinfection, removal of colour and humic substances (ultrafiltration) and removal of protozoan cysts (microfiltration) (Madaeni, 1999; Parsons and Jefferson, 2006).

Waste treatment. During the water treatment some unit processes produce considerable amount of waste sludge and wash-waters. These wastes need additional treatments before the disposal due to the potential for the transfer of pathogens.

Distribution. After treatment the water has to be conveyed to the consumer. This is done by a complex network of distribution pipes, also known as water mains, which are laid underground normally under roads and pavements. However, apart of water mains there are other important components of the DWDS. Basic components include valves, fittings, pumps, fire hydrants, storage reservoirs, tanks and the plumbing material in domestic, industrial and public buildings. Storage reservoirs are needed primarily because the water

resource and the water treatment plants are usually at considerable distances from the centre of population. They also had other important functions such as providing a reserve storage capacity in case of problems at the treatment plant or with trunk mains. There are two main categories of water main: the trunk and distribution mains. The trunk mains are the largest and do not have any branch or service pipe connections. They are used for transporting large volumes of water from the source to the treatment plant, from the plant to the service reservoir, and from one reservoir to another. The distribution mains consist of a pipe network of smaller, varying sized pipes, which is highly branched. It is the distribution main that supplies individual houses. The pipes come in a variety of materials. The most commonly used are iron (cast, spun or ductile), asbestos cement, uPVC (unplasticized polyvinyl chloride) and also mDPE (medium density polyethylene) (Gray, 2008). Owing to the effects on water quality, asbestos cement pipes are no longer installed and are being replaced by plastic pipes whenever possible. Another category of pipes are the service pipes, which conveys the water from the mains to the consumer's house. Service pipes can be made of mild steel, wrought iron, copper, lead, polyethylene (PE) or uPVC. The nature of the pipes that makes up the distribution system can affect the nature of the finished water as it travels from the treatment plant to the consumer's tap often altering its aesthetic and health-related quality. The materials used in the manufacture of the water distribution pipes have the most significant effect on quality. Aesthetic problems arise from sediment, discolouration, as well as odour and taste, which are often related to the microbial biofilms that grows on the internal surface of the pipes. These biofilms also support a range of larger organisms that occasionally appear in tap water, but the biofilm can affect the microbial quality of the water by allowing both pathogens and opportunistic bacteria to regrow (Gray, 2008).

When the water leaves a treatment plant, the water quality may be acceptable. However, a variety of physical, chemical and biological transformations can happen once the water enters and travel through a distribution systems. So, the water producers need to understand the several sources of water quality degradation in distribution systems in order to prevent their degradation and ensure the water that reaches consumers is safe.

2.2 Drinking water quality and public heath

The provision of safe DW is considered a top priority issue in any civilized society because safe DW is a basic need to human development, health, and well-being. Microbiologically and chemically contaminated DW has been linked with several health problems, e.g. cholera and typhoid fever are diseases associated with microbiologically contaminated DW (Hurst *et al.*, 2002; WHO, 2004).

It is important to guarantee a constant pressure of freshwater, but also high levels of quality. The quality of freshwater used for DW production is continually decreasing because the increasing of water utilization by the several sectors (population, agriculture and industry) and their continuous release on environment as contaminated water. So, as lower is the quality of freshwater, greater is the number of treatments necessary for their purification and consequently higher are the final costs. On the other hand the degradation of freshwater quality can also compromise the water treatment and consequently the quality of DW that arrive to the population.

The consumption of contaminated DW by the population can cause a wide range of diseases and health-related problems in all people or in some people more susceptible like infants, young children, elderly or people sick or immuno-compromised. The quality of DW is a universal public health concern. Diseases related to contamination of DW constitute a major burden on human health. So, any intervention to improve the quality of DW provides significant benefits to public health.

The introduction of water quality control plans is an effective way of ensuring that DW is safe for human consumption. Such plans are based on quality assessment and quality management of the entire supply chain from catchment to consumer. These include good management practices to minimize contamination of water resources, removing contaminants through effective and appropriate treatment, and preventing further contamination in the distribution network (DWI, 2005; Gray, 2008).

2.2.1 Drinking water quality problems

The DW contamination can occur in any point of the supply chain. The problems can arise from the resource due to leaching from natural rocks and soils or from chemicals used by man that subsequently enter the hydrological cycle, from the treatment plant, as the water travels from the treatment plant to the consumer via the distribution network or within the household plumbing system (Table 2.5).

Table 2.5 Main sources of DW contamination along of supply chain (based on Gray, 2008)

Resource	Water treatment	Distribution network	Home plumbing system
Natural geology	Unit process efficiency	Material of pipework, coating	Materials of pipework or tank
Land use	Chemicals added to clarify water	Organisms	Contamination
Pollution	Chemicals added for consumer protection	Contamination	Poor installation

According to Gray (2008), the most important DW quality problems arising along of supply chain in each point can be summarized in the Table 2.6.

Table 2.6 Source of principal DW quality problems (based on Gray, 2008)

Resource	Water treatment	Distribution network	Home plumbing system
Nitrate	Aluminium	Sediment	Lead
Pesticides	Discolouration	Discolouration	Copper
Industrial solvents	Chlorine	Asbestos	Zinc
Odour and taste	Odour and taste	Odour and taste	Odour and taste
Iron	Iron	Iron	Fibers
Manganese	Trihalomethanes	PAHs[c]	Corrosion
Pathogens	Pathogens	Pathogens	Pathogens
Hardness	Fluoride	Animals	
Algal toxins	Nitrite	Biofilm	
Radon/radionuclides	Acrylamide		
Arsenic			
PPCP[a]			
EDCs[b]			

[a] Pharmaceutical and personal care products; [b] Endocrine-disrupting compounds; [c] polycyclic aromatic hydrocarbons

The critical health issues relating to DW are infectious diseases, cancer, endocrine-disrupting compounds and fertility, mineral content, metals and organic compounds. While there are also aesthetic quality problems such as taste, odour and staining, which can be very problematic for consumers, it is the problems that result in a risk to health that must take

priority. The most important water-associated health problems in developing countries are waterborne diseases, especially those leading to diarrhoea, which is suspected of being responsible for between 3 to 5 million deaths per year, especially among young children. Control of pathogens in DW is comparatively straightforward, but poverty combined with water scarcity is a devastating combination. However, adequate supplies of clean water, combined with adequate sanitation and improved hygiene standards, would significantly reduce the incidence of waterborne disease, and especially diarrhoea, in developing countries. So, the reduction of pathogens in DW has been the priority for many decades. However, in recent years there has been a growing awareness of contamination from naturally occurring chemicals in groundwater and also from anthropogenic activities involving agriculture, industry and urban development (Gray, 2008).

The contaminants and pathogens associated with DW are very diverse in terms of their health effects, the time it takes for symptoms to develop and those who are most at risk. For example, the effects range in severity from minor conditions such as dental fluorosis to very severe life-threatening conditions that include birth defects and cancer. Many effects are rapid such as diarrhoea or methaemoglobinaemia, while some may take weeks (e.g. infectious hepatitis) or even years (e.g. cancer) to develop. Adverse effects can be caused by a single exposure, as with pathogens, or only after prolonged exposure, which is the case with most chemicals. Young children and the elderly are most at risk from waterborne diseases and chemical contaminants in DW. However, cryptosporidiosis, normally a mild and self-limiting infection, causes a high mortality for those with HIV, while the hepatitis E virus has a high mortality amongst pregnant women. While there is a need to quantify the risks associated with all water-related diseases and contaminants, it is extremely difficult to compare such diverse hazards with very diverse health outcomes. Exposure to contaminants occurs not only through DW but also from food, skin contact and inhalation. This varies between countries and is also affected by cultural and dietary habits. Many volatile substances in water will be released into the atmosphere during use, especially showering, while some may be absorbed during washing or bathing. The bacterium *L. pneumophila* and asbestos fibres both attack the lungs and so require to be inhaled rather than ingested, which most likely occurs during showering with contaminated water (Gray, 2008).

Although new issues and problems will come to light in the future, we will continue to see an improvement in water quality. Safeguarding DW quality is a shared responsibility

between those who use and dispose chemicals, who treat and supply water, and all of us who use it. The understanding of the problems, and an acceptance of that responsibility by all, will be needed if we are going to preserve one of our greatest resources for future generations: clean, safe DW on tap.

2.2.2 Drinking water quality control

A sufficient quantity of clean water is the prerequisite to good health and without it humans become susceptible to a surprising wide range of diseases and health-related problems. There are many countries in the world today where water scarcity, rather than quality, is the major issue in relation to health. Access to adequate and safe DW should be a basic human right, yet today there are 1.1 billion people globally that do not have access to sufficient safe DW. Many of these are managing on as little as five litres a day for all their drinking, washing and cooking needs. DW quality, especially in terms of pathogens, cannot be isolated from sanitation, with a total of 2.6 billion people currently lacking adequate sanitation facilities. The various health problems created by the lack of access to clean DW and proper sanitation is having a daily impact on 50% of the population of developing countries (UNDP, 2006).

The realization of high DW quality requires integrated control measures at all points along the supply chain starting with catchment management and the protection of water resources, throughout treatment, storage and distribution, as well as the home plumbing system. Thus, maintaining high quality DW is expensive, and may at times be unnecessary where no threat to human health has been identified. Therefore, DW standards must be a compromise between cost and risk to both consumers and the environment.

The WHO has proposed a preventative management framework to ensure safe DW (Figure 2.4). This comprises the following components: health-based targets; assessment of the supply system to ensure that targets can be met on a continuous basis; operational monitoring; assessment and monitoring procedures within a management plan that also incorporates operational and emergency procedures; and finally independent surveillance of the entire system, which feeds back to all the other components of the framework. Also, included within this management framework is the constant revision of the published health-based literature in relation to DW quality and the effects of individual substances and

pathogens found in water (Gray, 2008). This water safety management framework is universally being adopted both by rich and poor countries alike.

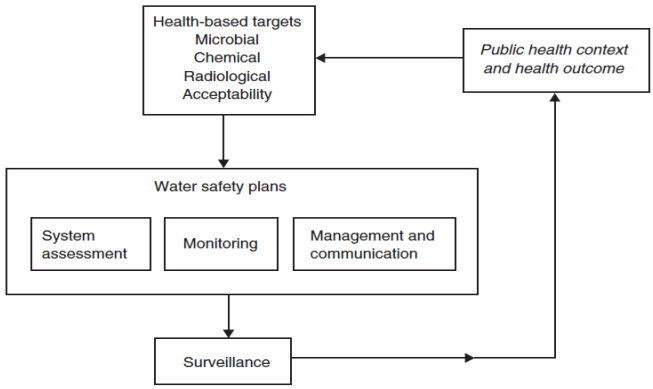


Figure 2.4 The WHO framework for safe DW (adapted from WHO, 2004).

Water safety plans have been used for many years to improve water quality control strategies, in conjunction with personal hygiene, to deliver sustainable health gains within the population (WHO, 2004). These plans use a combination of risk assessment and risk management techniques such as a multi-barrier approach to control pathogens and hazard analysis critical control points (HACCP) principles that were employed primarily by the food industry (Rasco and Bledsoe, 2005). While water safety plans are principally used to achieve health-based targets in developing countries, they equally apply to good water management practices and quality assurance systems (e.g. ISO 9001:2000) used in developed countries. Water safety plans protect public health by ensuring safe palatable water through good management practice. This includes the minimization of contamination of water resources, the removal or reduction of contaminants by appropriate treatment, and subsequent prevention of contamination within the distribution mains and the household plumbing system of the consumer.

Water safety plans are pivotal in protecting water resources and ensure safe and continuous supplies of drinking water to consumers. The water safety plan provides a framework that allows hazards to be identified, their risk to be assessed and then for a risk management protocol to be developed that includes control measures, the development of monitoring, incident and emergency plans, and the gathering of the necessary information about the operation and management of the water supply chain. As outlined in Figure 2.4, a water safety plan has three key components that are directed by health-based targets and overseen by a surveillance programme (WHO, 2004). Those are system assessment, operational monitoring, and management and communication. The development of a water

safety plan requires the selection of appropriate control measures to reduce or eliminate the risks identified. The importance of the catchment management has long been recognized in preventing contamination of surface and ground water resources that may be used for supply. Water treatment has to be designed specifically for each raw water supply and the potential risks identified. The control measures required can be summarized as the correct selection and optimal operation of the unit processes required to deal with the risks and contaminants identified, the use of approved water treatment chemicals and materials, and the use of reliable monitoring and back-up systems including alarms. Water entering the distribution system must be microbially safe, have a low concentration of dissolved organic matter and be non-corrosive. The distribution system must be operated to minimize sudden changes in flow, to prevent the natural detachment of biofilm and associated microorganisms, and the resuspension of solids, which can be controlled by routine flushing. Adequate positive pressures must be maintained at all times as pressure failure is the single most serious potential threat to water safety. A disinfectant residual must also be maintained throughout the network, where practicable, to control microbial regrowth in the water and on pipe surfaces. Protocols are required for carrying out repairs and dealing with incidents and emergencies. Appropriate disinfection is required before reconnection to supply where the network has been opened (Gray, 2008).

2.2.3 Drinking water microorganisms and waterborne diseases

The concept of safe DW on tap is a luxury not shared by the majority of the world's population and taken for granted by the majority of those who have it. More than a billion people have no access to safe DW, and over the past 20 years over 2 million people, mainly children, have died unnecessarily each year from water-related diarrhoea. In the developing world it is estimated that 45% of all deaths are due to contaminated DW. In these affected countries chemical quality is insignificant compared to the need for pathogen-free water to drink. Safety in this context is relative, and the success in preventing waterborne diseases in the developed world has focused attention on other contaminants. Yet, the risk from microbial pathogens remains ever present in the developed world and a daily challenge for the water treatment engineer and scientist.

Waterborne diseases are one of the major important water-associated health problems. Waterborne diseases are any illness caused by DW contaminated by human or

animal faeces, which contain pathogenic microorganisms. Waterborne pathogens are disease-causing bacteria, protozoa, and virus that are transmitted to people when they consume untreated or inadequately treated water and are listed in Table 2.7.

Table 2.7 Pathogens associated to waterborne diseases (Ashbolt, 2004; WHO, 2006; Karanis *et al.*, 2007).

Bacteria	Protozoa	Virus
Pseudomonas aeruginosa	*Balantidium coli*	Rotavirus
Legionella pneumophila	*Giardia lamblia*	Calicivirus
Aeromonas spp.	*Cryptosporidium parvum*	Norovirus
Mycobacterium spp.	*Entamoeba histolytica*	Astrovirus
Campylobacter jejuni	*Acanthamoeba castellani*	Adenovirus
Escherichia coli	*Naegleria fowleri*	Hepatite A virus
Yersinia enterocolitica	*Cyclospora cayetanensis*	Hepatite E virus
Helicobacter pylori	*Toxoplasma gondii*	Enterovirus
Salmonella spp.		Coxsackie virus
Shigella spp.		Echovirus
Vibrio cholera		Poliovirus
Leptospira spp.		
Burkholderia pseudomallei		
Toxic *cyanobacteria*		

If these pathogenic microorganisms are not removed by water treatment and disinfection and reach the consumer's tap, then they may cause outbreaks of disease within the community. Table 2.8 shows some outbreak diseases in several parts of the world caused by the consumption of contaminated water in the last 150 years.

Although gastroenteritis is the most known disease caused by waterborne outbreaks in developed countries, there are many others as reported elsewhere (Ashbolt, 2004): cholera, typhoid fever, meningitis, encephalitis, dysentery, hepatitis, legionellosis, pulmonary illness, poliomyelitis, leptospirosis, giardiasis and salmonellosis. These diseases were caused by the ingestion of pathogens-contaminated water or by the inhalation of aerosols with *Legionella* spp. and *Naegleria fowleri*.

Poor water quality continues to pose a major threat to human health. Diarrhoeal disease alone amounts to an estimated 4.1% of the total daily global burden of disease and is responsible for the death of 1.8 million people every year (WHO, 2004). It was estimated that 88% of that burden is attributable to unsafe water supply, sanitation and hygiene and is mostly concentrated on children in developing countries. Reduction in water-related

diseases could be prevented especially in developing countries through better access to safe water supply, adequate sanitation facilities and better hygiene practices.

Table 2.8 Some outbreak of diseases due to contaminated water supply (Bowen and McCarthy, 1983; Egoz *et al.*, 1988; Hayes *et al.*, 1989; Arnow *et al.*, 1994; Penman *et al.*, 1997; CDC, 1998; Kuusi *et al.*, 2004, 2005; Hrudey and Walker, 2005; Nygård *et al.*, 2006; Vestergaard *et al.*, 2007)

Year	Disease outbreak	City/Country	Additional information
1854	Cholera outbreak	London, England	Identified by Dr. John Snow as originating from contaminated water from the Broad street pump. This can be regarded as a founding event of the science of epidemiology
1980	Hepatitis A	Pennsylvania, USA	Consumption of water from a feces-contaminated well
1987	Cryptosporidiosis outbreak	western Georgia	Caused by the public water supply of which the filtration was contaminated
	Fluoride intoxication	Chicago, USA	Occurred in a long-term hemodialysis unit of university hospital due to the failure of a water deionization system
1988	Aluminium sulphate poisoning	Camelford, England	Many people were poisoned, when a worker put 20 tonnes of aluminium sulphate in the wrong tank
1993	Fluoride poisoning outbreak	Mississippi, USA	Resulting from overfeeding of fluoride
1993	*Cryptosporidium* outbreak	Milwaukee, USA	
	Typhoid fever outbreak followed by a large outbreak of dysentery	Israel	Outbreaks in north of Israel, associated with the contaminated municipal water supply
1997	*Cryptosporidium* outbreak	Minnesota, USA	Occurred 369 cases of cryptosporidiosis, caused by a contaminated fountain in the Minnesota zoo. Most of the sufferers were children
1998	Campylobacteriosis outbreak	Finland	A non-chlorinated municipal water supply was blamed for a campylobacteriosis outbreak in northern Finland
2000	Gastroenteritis outbreak	Finland	This outbreak was brought by a non-chlorinated community water supply, in southern Finland
2000	*E. coli* outbreak	Walkerton, Ontario, Canada	Seven people died from drinking contaminated water. Hundreds suffered from the symptoms of the disease, not knowing if they too would die
2004	Waterborne giardiasis outbreak	Bergen, Norway	Contamination of the community water supply
2007	Gastroenteritis outbreak with multiple etiologies	Denmark	Contaminated drinking water

Although the referred water-related diseases are rarely deadly in the developed countries, there are several authors reporting that DW consumption has several health risks attributed to pathogenic bacteria, protozoa and virus (Payment, 1999; Barbeau *et al.*, 2000; Gofti-Laroche *et al.*, 2003a; Gofti-Laroche *et al.*, 2003b; Exner, 2004; Paterson, 2006). Indeed, several outbreaks with public health risks occurred due to a malfunctioning of the DW treatment plant and distribution networks, which failed in maintaining a disinfectant residual to prevent growth of pathogens or were contaminated with pathogens sources. In this millennium, some reported waterborne outbreaks were due to *E. coli* O157:H7,

Campylobacter spp., *H. pylori*, *P. aeruginosa*, *Cryptosporidium parvum* and virus (*e.g.*, norovirus, calcivirus, enterovirus) in Canada, France, England, Finland, Switzerland, Northern Ireland, Russia, New Zealand and Poland (Hafliger *et al.*, 2000; Glaberman *et al.*, 2002; Hanninen *et al.*, 2003; Said *et al.*, 2003; Amvrosy'eva *et al.*, 2004; Laporte *et al.*, 2004; Maunula *et al.*, 2005; Garg *et al.*, 2006; Empel *et al.*, 2007; Hewitt *et al.*, 2007).

Despite of the numerous reported outbreaks, these numbers are surely underestimated as not all outbreaks are recognized, investigated or reported. Nowadays these health risky events are still occurring too frequently as several research groups emphasized (Blackburn *et al.*, 2004; Afzal, 2006; Liang *et al.*, 2006): 31 events in 2000-2001 and 30 events in 2003 - 2004 in the USA. Craun *et al.* (2006) reported that during 1920 to 2002 at least 1870 outbreaks were associated with DW, an average of 22.5 per year and 883806 illnesses, an average of 10648 cases per year. These outbreaks were attributed to microbial contaminations with virus, parasites and pathogenic bacteria or to chemical/toxin poisonings.

Recognized harbours of pathogenic microbial contaminants in DWDS are biofilms that develop in the pipes of the distribution network. In these microbial consortiums, pathogenic microorganisms are protected of stress conditions (chlorine, shear stress, temperature), which allows their maintenance in a viable state. So, when the detachment of portions of these biofilms occurs they enter into the bulk water, originating a possible outbreak of pathogens. It is well known that biofilms constitute one of the major microbial problems in DWDS that most contributes to the deterioration of water quality. However, their elimination from these systems is almost impossible, but several aspected can be considered in order to prevent and control their growth.

2.3 Biofilms in drinking water distribution systems

The biofilms in DWDS, also designated as biofouling in DWDS, has been studied in an intensive way in the last decades. Therefore, there is a wide range of reviews published on this topic covering aspects such as hygienic quality of DW (Whipple, 1897; Prescott and Winslow, 1904; Hastings, 1948; Geldreich, 1974; Allen, 1979; Reasoner, 1983; Anon, 1994; Walker and Morales, 1997; Momba *et al.*, 2000; Szewzyk *et al.*, 2000; Bachmann and Edyvean, 2005; Skraber *et al.*, 2005; Dietrich, 2006), its development (Olson *et al.*, 1991; Block *et al.*, 1994; Van der Kooij and Veenendaal, 1994, Camper *et al.*, 1999; Momba *et al.*, 2000; Bachmann and Edyvean, 2005) and control (Walker *et al.*, 1997; Van der Kooij *et al.*, 1999; Bachmann and Edyvean, 2005).

Biofouling, in general, refers to the undesirable accumulation of biotic matter on a surface. It has been shown to be of considerable hygienic, operational and economical relevance, not only in DWDS but also in other purified water supply systems such as dental unit waterlines (Pankhurst *et al.*, 1998; Walker *et al.*, 2001; Franco *et al.*, 2005), dialysis units (du Moulin *et al.*, 1987; Pontoriero *et al.*, 2003), laboratories (McFeters *et al.*, 1993), reverse osmosis (Flemming, 1997), pharmaceutics (Riedewald, 1997), the semiconductor industry (Harned, 1986; Kim *et al.*, 1997), and even the International Space Station Water Recovery and Management system (Roman and Minton-Summers, 1998).

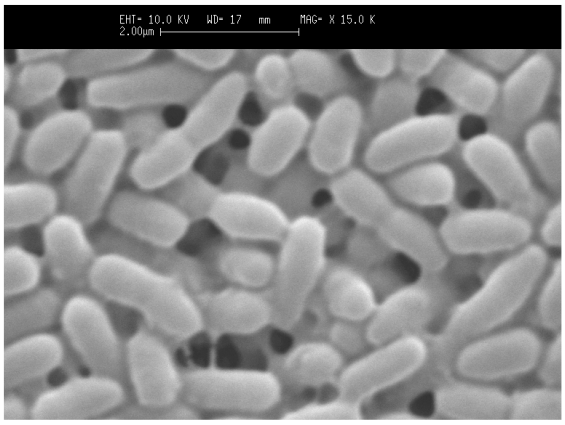
2.3.1 Biofilm definition and their impact

Biofilm science is a relatively new technical discipline focused on the understanding and modulating of the combination of biological and chemical processes as well as in the transport and interfacial transfer processes that potentially affect the microbial accumulation and activity on both biotic and abiotic surfaces. Research on biofilms has progressed rapidly in the last decade. Due to the fact that biofilms have required the development of new analytical tools, many recent advances have resulted from collaborations between microbiologists, microbial ecologists, medical doctors, pharmacologists, engineers and mathematicians. The scientific community has come to understand many things about the

particular aspects of microbial biofilms through a variety of microscopic, physical, chemical, and molecular techniques of study.

Bacteria are able to adapt to undesirable changes in nutrient availability, environmental conditions and presence of antimicrobial products, as well as to immunological defenses. One particularly important example of bacterial adaptation is the ability to grow as part of a sessile community, commonly referred to as biofilm. It is a natural tendency of microorganisms to attach to wet surfaces, to multiply and to embed themselves in a slimy matrix composed of extracellular polymeric substances (EPS) that they produce, forming a biofilm (Figure 2.5a). These are well organized structures where microorganisms are protected from environmental stress and allow complex interactions among different species, i.e. antagonistic or synergistic relationships (Nielsen *et al.*, 2000; Christensen *et al.*, 2002; Rao *et al.*, 2005; *et al.*, 2007b). In biofilms, the way that cells communicate and organize in a social community is controlled by the secretion of signal molecules in a process called "quorum sensing". These promotes the communication between cells and regulates the relationship between cell resulting in a group behaviour instead of an individual performance (Watnick and Kolter, 2000; Daniels *et al.*, 2004; Parsek and Greenberg, 2005). It is supposed that biofilm is the first form of communitarian life recorded on the planet, being estimated that most microorganisms on Earth are organized in biofilms and they even occur in extreme environments such as hydrothermal vents, nuclear power plants and disinfection pipelines (Costerton *et al.*, 1987).

Concerning discover of biofilms, it has been first documented in 1943 by Zobell, who reported the attachment of layers of microbial cells to bottle walls and the increase in the biological activity of batch suspended cultures when glass rods were added (Zobell, 1943). Further investigations revealed that this effect was even more pronounced under oligotrophic conditions when compared to the results obtained under high nutrient conditions (Heukelekian and Heller, 1940; Zobell, 1943). These conclusions emphasized the perception that the adhesion is a strategy of microorganisms to access nutrients from the surface and biofilm. The study of Characklis (1973), about microbial slimes in industrial water systems, revealed their high cohesiveness as well as their strong resistance to disinfectants, but it was Costerton *et al.* in 1978, which postulated the general theory of biofilm predominance. Only recently, however, have attempts been made to define genetic, physiological and ecological basis of such phenomena (O`Toole *et al.*, 1999; Davey and O`Toole, 2000; Battin *et al.*, 2007).



(a) **(b)**

Figure 2.5 (a) Scanning electron microscopy (SEM) photomicrographs of 24 hours old biofilms formed by the opportunistic Gram-negative *Burkolderia cepacia* (isolated from laboratorial DWDS) evidencing the presence of an extracellular polymeric matrix (× 15000 magnification; bar = 2 µm). (b) Ductile iron pipe section from a DWDS with biofilm and high amounts of corrosion products.

In general, a biofilm can therefore be defined as a community of microorganisms that is irreversibly attached to a biotic or abiotic surface and that is enclosed in a matrix of exopolymeric products (Costerton *et al.*, 1999; Prakash *et al.*, 2003). DW biofilms, particularly, are composed by complex microbial communities functionally organized and embedded in a gelatinous matrix of extracellular polymers excreted by microorganisms. Any inorganic particle passing nearby (e.g. corrosion products, clays, sand…) may also be incorporated in the biofilms (Figure 2.5b) which is known to increase its "mechanical strength" (Vieira and Melo, 1995). According to Characklis and Marshall (1990), bacteria are generally dominant in whatever biofilm due to their high growth rates, low sizes, adaptation capacity and the ability for to prodice extracellular polymers. However, virus, protozoa, fungi and algae may also be present in DW biofilms as reported by several authors (Momba *et al.*, 2000; Codony *et al.*, 2003; Snelling *et al.*, 2006).

Biofilms are formed ubiquitously in any interface (liquid or solid) in contact with water. The ubiquity of biofilms can cause significant problems of public health, medicine and industry concern (Costerton *et al.*, 1987; Donlan and Costerton, 2002; Hall-Stoodley *et al.*, 2004; Raad *et al.*, 2007). Accordingly, there has been a great deal of research to better understand biofilm development and to identify improved control strategies. In the health context, some diseases and adverse medical conditions are now recognized to be the result of a biofilm infection.

Biofilms are as versatile as they are ubiquitous. Intentional and unintentional biofilms concern a broad range of areas, comprising special attention in the industrial, environmental and biomedical areas (Bryers, 2000). Biofilms can be beneficial or detrimental depending where they are found. Biofilms used in pharmaceutical and food fermentation industries, wastewater treatment plants and natural biofilms presents in lakes or rivers which contribute to pollutant degradation are examples of some beneficial biofilms. On other hand, biofilm that accumulate in cooling water towers and heat exchangers, membrane systems, filters, DWDS, swimming pools, food processing equipment, paper manufacture industries, ship hulls, catheters, medical implants, tissues, teeth and contact lenses are harmful. One of the main problems of these biofilms is their potential impact in human health. Moreover, they may create problems for hygiene and cleaning, as well as being responsible for energy losses, blockages in systems and microbial induced corrosion.

2.3.2 Relevance of biofilms in the water industry

Many problems in DWDS are microbial in nature, including biofilm growth, nitrification, microbially mediated corrosion, and the occurrence and persistence of pathogens (Regan *et al.*, 2003; Beech and Sunner, 2004; Camper, 2004; Emtiazi *et al.*, 2004). Biofilms are suspected to be the primary source of microorganisms in DWDS that are fed with treated water and have no pipeline breaches, and are of particular concern in older DWDS (LeChevalier *et al.*, 1987). Flemming *et al.* (2002) estimated that 95% of the overall biomass is attached to pipe walls, while only 5% is in the water phase. Therefore, the development of bacteria in biofilms is highly relevant for water quality since they may directly affect cell density in the bulk water phase.

By adopting this sessile mode of life, biofilm-embedded microorganisms enjoy a number of advantages over their planktonic counterparts. One advantage is the ability of the extracellular polymeric matrix, they excrete, to capture and concentrate a number of environmental nutrients, such as carbon, nitrogen and phosphate (Simões *et al.*, 2006). Another advantage to the biofilm mode of growth is that it enables resistance to a number of removal strategies, such as antimicrobial and antifouling agents and shear stress conditions (Simões *et al.*, 2005a; 2005b). DWDS disinfection with chlorine dioxide and chlorine, for example, can reduce the concentration of planktonic bacteria, but have little to no effect on

the concentration of biofilm bacteria (Gagnon *et al.*, 2005). This inherent resistance to antimicrobial factors is mediated through very low metabolic levels and drastically downregulated rates of cell division of the deeply embedded microorganisms. Furthermore, biofilms act as a diffusion barrier, slowing down the penetration, to some antimicrobial agents (Simões *et al.*, 2007c). Another advantage of living in a biofilm is the possibility of metabolic interactions between bacteria with different physiological requirements. This will promote the formation of different spatial niches in a biofilm in response to environmental conditions and the activity of their neighbors in order to optimize the nutritive resources (Tolker-Nielsen and Molin, 2000). Bacteria communication through excreted signalling molecules is another advantage of living in biofilm communities (Wuertz *et al.*, 2004). The last advantage to the biofilm mode of growth is the potential for dispersion *via* detachment. Under the direction of fluid flow, detached microorganisms travel to other regions to attach and promote biofilm formation on clean areas (Codony *et al.*, 2005). Therefore, this advantage allows a persistent bacterial source population that is resistant to antimicrobial agents, while at the same time enabling continuous shedding to promote bacterial spread.

The current knowledge of the structure and activities in biofilm communities stills limited, because analysis of microbial physiology and genetics have been largely confined to studies of microorganisms from few lineages for which cultivation conditions have been determined and for some process conditions, not mimicking real environments. The dynamics of the microbial growth in DW networks is very complex, as a large number of interacting processes are involved. DW pipes inner-surfaces are invariably colonized by biofilm, regardless of the presence of a disinfectant residual. In addition to the possibility of causing corrosion, taste and odour problems, biofilms control the microbiological contents of the distributed water and are a potential source of pathogens (Percival and Walker, 1999; Szewzyk *et al.*, 2000). The interaction of pathogens with biofilms has predominantly been a concern in man made water systems, particularly DWDS. In fact, biofilms formed within potable-water systems contain bacterial pathogens such as *L. pneumophila* and coliforms of intestinal and nonintestinal origin (WHO, 1993). Furthermore, protozoa are commonly found within water distribution systems and have been associated with the persistence and invasiveness of pathogens (Tyndall and Domingue, 1982). Despite Payment *et al.* (1993) in his work did not find any relationship between biofilm presence in DWDS and occurrence of disease, it has been proved that pathogens such as *L. pneumophila*, *Mycobacterium* spp., *P. aeruginosa*, *Klebsiella* spp., *Burkholderia* spp., *Giardia* and *Cryptosporidium*, among

others (Table 2.7), are transmitted by contaminated water and biofilms are a good candidate as they can act as a protective niche for their survival in DW as showed by several authors (Ford, 1999; Szewzyk *et al.*, 2000; Sharma *et al.*, 2003). Such findings implicate the importance of maintaining a continuous disinfectant residual in DWDS as the most usual strategy to control biofilm formation.

2.3.3 Biofilm formation

There are a number of mechanisms by which numbers of microbial species are able to come into closer contact with a surface, attach firmly to it, promote cell-cell interactions and grow as a complex structure (Bryers and Ratner, 2004). Biofilm formation comprises a sequence of steps (Bryers and Ratner, 2004). The biofilm formation mechanisms will only be described briefly, nonetheless there are several excellent comprehensive reviews on this area (O`Toole *et al.*, 2000; Donlan and Costerton, 2002; Hall-Stoodley and Stoodley, 2002; Chmielewski and Frank, 2003; Bryers and Ratner, 2004; Verstraeten *et al.*, 2008).

At present, processes governing biofilm formation that have been identified include the following steps (Figure 2.6) (Bryers and Ratner, 2004; Simões *et al.*, 2009):

1. Preconditioning of the adhesion surface either by macromolecules present in the bulk liquid or intentionally coated on the surface;

2. Transport of planktonic cells from the bulk liquid to the surface;

3. Adsorption of cells at the surface;

4. Desorption of reversibly adsorbed cells;

5. Irreversible adsorption of bacterial cells at a surface;

6. Production of cell-cell signalling molecules;

7. Transport of substrates to and within the biofilm;

8. Substrate metabolism by the biofilm-bound cells and transport of products out of the biofilm. These processes are accompanied by cell growth, replication, and production of EPS;

9. Biofilm removal by detachment or sloughing.

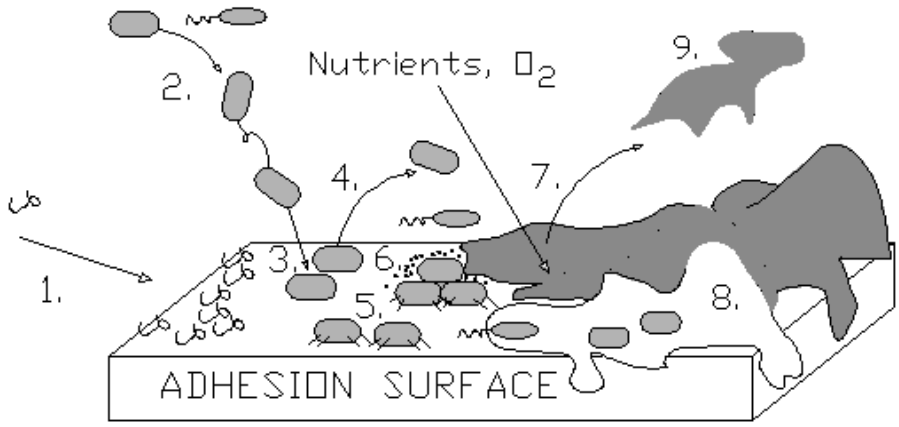


Figure 2.6 Processes governing biofilm formation (adapted from Simões *et al.*, 2009).

Conditioning film. The first step of biofilm formation is the preconditioning of the adhesion surface. The conditioning film is a thin layer of organic molecules and ions covering the adhesion surface that is formed before any microorganisms attach to the surface. These molecules may adhere to the surfaces by physical or chemical adsorption. Physical adsorption is generally a reversible process in which one monolayer is formed, involving nonspecific bonds (London and van der Waals). In chemical adsorption, several adsorbed molecular layers are formed and involves specific chemical bonds (electrostatic, covalent and hydrogen bonds), dipole interactions, and hydrophobic interactions (Marshall, 1996). The strength of biofilm adhesion is largely dependent on the cohesion of the conditioning as observed by several authors (Busscher *et al.*, 1995; Bos *et al.*, 1999).

Adhesion. The steps between 2 and 5 correspond to the effective adhesion of bacteria to surfaces. This is started by the transport of microbial cells to the adhesion surface either by fluid dynamics, gravitational forces and Brownian motion, or by migration through active cell motility (e.g. flagella). Also, the surface electrostatic charge and hydrophobic interactions affect this approaching and the adhesion process. When the cells approach the surface they can interact with each other by the establishment of long and short/intermediate distance forces. The long distance forces are described by the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory and comprise the attractive forces of van der Waals and the repulsive forces of the electrostatic double-layer. In equilibrium, when favourable, this results in the adhesion of microorganisms. The short/intermediate distance forces include hydrophobic interactions, hydrophobic pressure, steric forces, Born repulsion forces and polymer bridges (Freter *et al.*, 1984; Oliveira, 1992).

Biofilm growth and maturation. After cellular adhesion to surfaces, the growth and maturation are the following stages of biofilm formation (steps 6-9). The attached bacteria start growing, they form microcolonies, excreting organic polymers and initiating the formation of the biofilm matrix. Exopolysaccharide synthesis has been shown to be important for the formation of microcolonies (Allison and Sutherland, 1987; Watnick and Kolter, 1999). As biofilm thickness increases, transport of nutrients from the external liquid media to the inner layers of biofilm and transport of excreted metabolites in the opposite direction are important for biofilm maintenance. Throughout the phase of biofilm growth, bacteria detachment events occur although at a lower extent compared to the growth rate. In the maturation phase, there are the development of a complex and organized consortia of microorganisms embedded in an organic matrix that protects the microorganisms inside from stress factors. It is in this stage that microorganisms produce large amounts of EPS. The structure of a mature biofilm depends on the microbial composition, EPS production, the nutrient availability, hydrodynamic conditions and temperature. In a mature biofilm several processes may occur simultaneously: bacteria detachment into water, attachment of planktonic bacteria, growth and death. However, in this stage these processes are at equilibrium and the attached cells per unit surface area are constant with time, although with periodic fluctuations (Vieira *et al.*, 1993; Bryers, 2000). At this phase, the biofilm should reach the highest thickness that is essentially dependent on the hydraulic conditions, the mass transport and the biofilm cohesion.

Detachment. The last phase of biofilm formation (step 9) is the detachment of cells and other components from the biofilm. Hydraulic shear stress provoked by high flow velocities can lead to detachment of bacteria and biofilm aggregates (sloughing), with higher detachment rates at increasing shear (Characklis *et al.*, 1990). Detachment occurs due to different mechanisms: erosion (the continuous release of single cells or small clusters of cells), sloughing (the rapid detachment of large portions of the biofilm), abrasion (collision of solid particles with the biofilm), and predator grazing. Erosion and sloughing can result from biofilm-associated processes, such as enzyme production (Lee *et al.*, 1996; Allison *et al.*, 1998), the excretion of certain signaling molecules (Wuertz *et al.*, 2004), cell-cycle-mediated events (Allison *et al.*, 1990; Gilbert *et al.*, 1993), and the excretion of surface modified products (surfactants) by certain bacteria (Rosenberg and Ron, 1999), or from external factors such as shear forces (Gilbert *et al.*, 1993; Picioreanu *et al.*, 2001), variations in the nutrient concentration (Sauer *et al.*, 2004), a chemical change in EPS due to the

presence of chelating agents (Ca^{2+}) that will reduce the cohesive strength of the attached cells (Chen and Stewart, 2002), abrasion, and predator grazing (Stewart, 1993).

2.3.4 Biofilm structure and composition

The knowledge of biofilm structure allows a better understanding of how developing biofilms are influenced by the surrounding environment and affords better interpretation of biofilm processes. Over time there has been a shift on perception of the structure of microbial biofilms from that of a homogenous layer of cells in a slime matrix to a much more heterogeneous arrangement.

So, several structures have been proposed as biofilm visualizing techniques were improved through the years.

The first simplifying assumption that probably extended through the 1980's well into the following decade was that a biofilm could be represented as a simple planar structure, largely 2D, with a relatively constant thickness (Wimpenny *et al.*, 2000).

In the meantime episcopic differential interference contrast microscope was developed by Keevil and Walker (1992) and the heterogeneous mosaic model was proposed for biofilms growing on the inner surfaces of DWDS (Figure 2.7a). These researchers discerned stacks consisting of microcolonies of bacteria held together by EPS and appearing as columns surrounded by a liquid phase in which grazing protozoa could be discerned. Below the stacks there was a layer of cells about 5 µm thick attached to the substratum. These types of structure led Bill Keevil to name this the “heterogeneous mosaic model” (Figure 2.7a).

Another biofilm structure was proposed by Costerton and co-workers (Lawrence *et al.*, 1991; Costerton *et al.*, 1994; Stoodley *et al.*, 1994; James *et al.*, 1995). When working with river biofilms supplemented with nutrients, these researchers observed a heterogeneous structure composed of mushrooms with the stalk narrower than the upper surface parts, the whole being penetrated by channels allowing the transportation of water, nutrients and metabolites (Figure 2.7b).

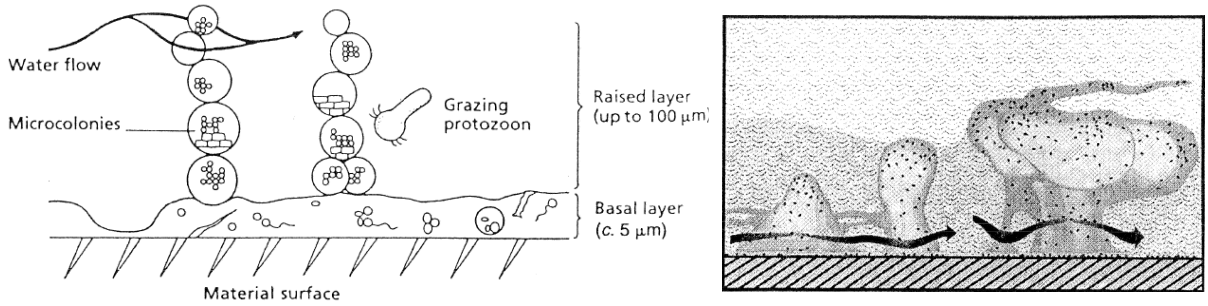


Figure 2.7 (a) Heterogeneous mosaic biofilm model according to Keevil *et al.*, 1995 and Walker *et al.*, 1995. (b) Water channel model according to Costerton and co-workers (adapted from Wimpenny and Colasanti, 1997).

According to Wimpenny and Colasanti (1997), who proposed a unifying hypothesis for the microbial biofilm structure based on simple and automaton model, all these conceptual structure models were correct since the final structure was largely dependent on the resource concentration. Thus, the first type was dense relatively uniform biofilm found in habitats (e.g. the human mouth) where the nutrient levels are generally high, or periodically extremely high. The second type appeared in water distribution systems where the substrate concentration is very low. The third type was generated in the laboratory using media containing significant nutrient concentration (Wimpenny, 2000). However, there are reports that indicate the presence of channels in dental plaque biofilms (Wood *et al.*, 2000) and describe a dense flat biofilm formed under conditions of phosphate starvation (Huang *et al.*, 1998).

The biofilm structure can be determined by a great variety of environmental parameters such as the hydrodynamics, the nutrient composition, the temperature and the pH that, consequently, affect the density, the porosity and the thickness (Wimpenny and Colasanti, 1997; Sutherland, 2001a; Horn *et al.*, 2002). Most environmental biofilms are heterogeneous microbial communities that have different behaviours depending on the conditions (e.g. exopolymers) and the interactions with each other (e.g. chemotaxis, metabolic interactions), hence forming unique biofilms where all resources and energies are optimized.

The structure of DW biofilms on the pipe surface does not follow a standard rule: they may cover the entire inner surface (Donlan, 2002; Chu *et al.*, 2003; Yu *et al.*, 2007) or be formed by dispersal aggregates (Keevil and Walker, 1992). The surface coverage degree

depend of many factors, such as the type of microorganisms, biofilm age, hydrodynamic conditions, presence of inorganic particles, nutrients and temperature.

As result of the application of advanced microscopy, such as confocal laser scanning microscopy and episcopic differential interference contrast microscopy, molecular and electrochemical high-resolution methods has provided insights into the structural organization and function of biofilm communities. Therefore, a mature biofilm is seen as very heterogeneous arrangement, with a basic community structure consisting of microcolonies of bacterial cells encased in EPS matrix separated by water channels (Lewandowski, 2000; Donlan and Costerton, 2002). But although some structural attributes can generally be considered universal, every microbial community is unique (Tolker-Nielsen and Molin, 2000). This is due to the fact that a biofilm structure can be influenced by several conditions, such as surface and interface properties, nutrient availability, the microbial community composition, and hydrodynamics, making the exact structure of any biofilm probably a sole feature of the environment in which it develops (Stoodley *et al.*, 1997; Stoodley *et al.*, 1999; Sutherland, 2001b). The water channels that separated the matrix enclosed microcolonies are vital for biofilm maintenance, providing a nutrient flow system within it (Donlan and Costerton, 2002), that delivers nutrients deep within the complex community (Stoodley *et al.*, 2002) and allows the exchange of metabolic products with the bulk fluid layer (Costerton, 1995).

Concerning the biofilm composition, water is considered to be the major component of the biofilm, representing from 70 to 99% while bacteria occupy only between 10 and 50% of the total volume of biofilm (Costerton *et al.*, 1995; Zhang *et al.*, 1998; Watnick and Kolter, 2000). EPS, the major component of biofilm matrix, are considered the organic substances excreted by attached microorganisms, account for 50 to 90% of the total organic carbon of biofilms (Flemming *et al.*, 2000) and are important keys for the biofilm start-up (Watnick and Kolter, 2000; Melo, 2003). Their composition and amount are highly influenced by the type of microorganisms and environmental conditions such as nutrients, temperature, pH and hydrodynamics. For example, the excess of available carbon and the limitation of other nutrients (nitrogen and phosphate), promoted exopolysacharides synthesis (Sutherland, 2001a; Melo, 2003). The EPS determine the structural and functional integrity of microbial biofilms, and contribute significantly to the organization of the biofilm community (Branda *et al.*, 2005). EPS are involved in the formation and

maintenance of a three-dimensional, gel-like, highly hydrated and locally charged biofilm matrix, in which the microorganisms are more or less immobilized.

Besides polysaccharides, proteins, nucleic acids or phospholipids, non-cellular materials such as mineral crystals, corrosion products or blood components, may also be found in the biofilm matrix (Donlan, 2002). The biofilm matrix (composed by all inorganic and organic substances surrounding the cells) has several functions. Furthermore, acting as the structural backbone, biofilm matrix protects bacteria from being washed out, from mechanical shocks, from toxic/lethal attacks by antibiotics (Stewart and Costerton, 2001), disinfection chemicals (LeChevallier *et al.*, 1988a; Costerton *et al.*, 1995), UV radiation (Hijnen *et al.*, 2006), predators (Keevil and Walker, 1992) and from desiccation (LeChevallier *et al.*, 1988a, 1988b; Sutherland, 2001a). As well promotes the storage of nutrients for intake during periods of limitation (Wolfaardt *et al.*, 1998), the retention of extracellular enzymes (Flemming, 2002), the horizontal gene transfer (Molin and Tolker-Nielsen, 2003), and the exchange of signaling molecules and metabolites (Wuertz *et al.*, 2004).

2.3.5 Factors affecting biofilm growth

The attachment of microorganisms to surfaces and the subsequent biofilm development are very complex processes, affected by several variables (Table 2.9). In general, attachment will occur most readily on surfaces that are rougher, more hydrophobic, and coated by surface conditioning films (Millsap *et al.*, 1997; Donlan, 2002; Chae *et al.*, 2006; Patel *et al.*, 2007; Oulahal *et al.*, 2008; Simões *et al.*, 2007a, 2008). Properties of the cell surface, particularly the presence of extracellular appendages, the interactions involved in cell-cell communication and EPS production are important for biofilm formation and development (Davies *et al.*, 1998; Sauer and Camper, 2001; Donlan, 2002; Allison, 2003; Parsek and Greenberg, 2005). An increase in flow velocity, water temperature or nutrient concentration may also equate to increased attachment, if these factors do not exceed critical levels (Vieira *et al.*, 1993; Stoodley *et al.*, 1999; Simões *et al.*, 2007c). Table 2.9 summarizes the main variables involved on cell attachment and biofilm formation. However, in real systems all these variables work together to influence biofilm accumulation. Thus, the impact of some of them may be insignificant comparatively with the impact of others and must therefore be considered carefully for each system.

Table 2.9 Variables important in cell attachment, biofilm formation and development (based on Donlan, 2002)

Adhesion surface	Bulk fluid	Cell
Texture or roughness	Flow velocity	Cell surface hydrophobicity
Hydrophobicity	pH	Extracellular appendages
Surface chemistry	Temperature	EPS
Charge	Cations	Signalling molecules
Conditioning film	Presence of residual disinfectants	
	Nutrient availability	

The main aspects, referred in Table 2.9, will be briefly described.

Support material. The variability of materials in DWDS is high. Formerly, the majority of pipelines in DW networks were made of iron-based or cement-based materials. More recently, polymeric materials have been preferred, mainly polyvinyl chloride (PVC), PE, because they are easier to handle and implement. In fact, it is possible to find all this types of materials in the same DWDS. The influence of support materials on biofilm growth is well documented in the literature (Schwartz *et al.*, 1998; Kerr *et al.*, 1999; Momba *et al.*, 2000; Niquette *et al.*, 2000; Cloete *et al.*, 2003). However, there is still controversy about the effects of surface materials on biofilm development when were compared polymeric and metallic materials. The main characteristics of materials that have been identified as important on biofilm formation are the roughness and the surface physicochemical properties (chemical composition, solid surface tension, hydrophobicity and surface charge).

Bulk fluid. pH and temperature are considered two important factors affecting life by modifying the electrostatic interactions between surfaces and microorganisms, enzymatic activity, kinetics and equilibrium of reactions, and other properties (e.g. diffusivity, solubility). Also, chlorine residuals present in DWDS are drastically reduced when temperature increases and pH decreases. The hydrodynamic conditions in DWDS range alternatively from laminar to turbulent flow, but stagnant waters also occur in places where the water consumption is low, as well as in reservoirs and buildings. The flow velocity may cause different effects on biofilm accumulation and detachment (Melo, 2003). Nutrients transport rates within the biofilm increased with the flow velocity until reaching maximum values, and then decreases as the velocities is further increased. This transport rate promotes bacterial growth within the biofilm. On the other hand, the biofilm density and detachment

increased with the flow velocity. Generally, DWDS are considered oligotrophic environments with low contents of nutrients (carbon, nitrogen and phosphorous). However, the increase of nutrients in water promotes biofilm formation. Studies have shown a positive relationship between the concentration of nutrients in DW and bacterial regrowth in DWDS (van der Kooij, 1992; Owen *et al.*, 1995). The hydrodynamic conditions and the nutrients are the two main parameters that influence biofilm growth in particular the structure, density and thickness (Wimpenny and Colasanti, 1997; Horn *et al.*, 2002). High shear stress and limitation of nutrients led to thin and dense biofilms that will have reduced internal nutrient diffusion (Melo and Vieira, 1999) and increased resistance to removal and cohesion (Vieira *et al.*, 1993; van Loosdrecht *et al.*, 1997; Chen *et al.*, 2005; Paris *et al.*, 2007). Under low flow velocities and high nutrients content, the biofilm grow quickly with a less dense structure with many pores, channels and protuberances (van Loosdrecht *et al.*, 1995).

Other important variable in biofilm formation is the concentration of disinfectant in DWDS. The chemical disinfection and the maintenance of chlorine residual through the distribution systems are worldwide strategy to prevent bacterial regrowth during the water transportation (LeChevallier *et al.*, 1996; Momba *et al.*, 1998; Codony *et al.*, 2005). Even so, the regrowth may occur when the chlorine residual decay further down in the distribution system (Chandy and Angles, 2001; Hallam *et al.*, 2002).

Cell. The physiological state and the type of microorganisms present in bulk water will affect the attachment process, since each microorganism has different surface properties, extracellular appendages and abilities to produce EPS. Cell surface hydrophobicity and the presence of extracellular filamentous appendages may influence the rate and the extent of microbial attachment. The hydrophobicity of the cell surface is important in adhesion because hydrophobic interactions tend to increase with an increasing non-polar nature of one or both surfaces involved, *i.e.*, the microbial cell and the adhesion surface (Donlan, 2002). According to Drenkard and Ausubel (2002), the ability of bacteria to attach to each other and to surfaces depends in part on the interaction of hydrophobic domains.

Many cells produce extracellular filamentous appendages. These may, therefore, play a role in the attachment process. In fact, their radius of interaction with the surface is far lower than that of the cell itself. A number of such structures are known to exist - *flagella*, *pili* or *fimbrae*, *prothecae*, *stalks* and *holdfast* (Harbron and Kent, 1988).

Flagella, when existent, are responsible for the motility of bacteria. These are very fine threads of the protein flagellin with a helical structure extending out from the cytoplasm through the cell wall. *Flagella* may have a diameter between 0.01 to 0.02 µm, and a length of up to 10 µm. Many types of bacteria have *flagella*. It is possible that the flagellum itself may form an adhesive bond with the adhesion surface (Harbron and Kent, 1988). The primary function of *flagella* in biofilm formation is assumed to be in transport and in initial cell-surface interactions (Sauer and Camper, 2001). *Flagella*-mediated motility is believed to overcome repulsive forces at the surface of the substratum and, as a consequence, a monolayer of cells forms on the adhesion surface (Daniels *et al.*, 2004).

Pili or *fimbriae* are found on many Gram-negative bacteria. They are fine, filamentous appendages, also of protein, 4 to 35 nm wide and up to several micrometers long (Harbron and Kent, 1988). These structures are usually straight, and are not involved in motility. Their only known general function is to make cells more adhesive, since bacteria with *pili* can adhere strongly to other bacterial cells and inorganic particles (Harbron and Kent, 1988). Nevertheless, they are not always involved in the attachment process even if they are present (Characklis and Cooksey, 1983). According to Sauer and Camper (2001), *pili* and pilus-associated structures have been shown to be important for the adherence to and colonization of surfaces, probably by overcoming the initial electrostatic repulsion barrier that exists between the cell and the substratum.

Prosthecae and *stalks* form a third group of attachment structures. These occur in several types of microorganisms. They may occur at one or more sites on the cell surface, and are filiform or blunt extensions (commonly 0.2 µm) of the cell wall and membrane (Harbron and Kent, 1988). At the end of a *prosthecae* or *stalk* is usually found an adhesive disk, or *hold-fast*. The *stalk* and *hold-fast* structure is quite often used by diatoms to attach to a surface (Harbron and Kent, 1988).

EPS are responsible for binding cells and other particulate materials together (cohesion) and to the surface (adhesion) (Characklis and Wilderer, 1989; Sutherland, 2001a; Allison, 2003). The general composition of bacterial EPS comprises polysaccharides, proteins, nucleic acids, lipids, phospholipids, and humic substances (Jahn and Nielsen, 1998; Wingender *et al.*, 1999; Sutherland, 2001a). According to Tsuneda *et al.* (2003), proteins and polysaccharides account for 75-89% of the biofilm EPS composition, indicating that they are the major components.

Biofilms form a gel phase where microorganisms live inside (Wingender et al., 1999; Sutherland, 2001a). The EPS matrix acts as a barrier in which diffusive transport prevails over convective transport (Sutherland, 2001a). A function frequently attributed to EPS is their general protective effect on biofilm microorganisms against adverse conditions. As an example, it has frequently been observed that biofilm cells can tolerate high concentrations of antimicrobials (Foley and Gilbert, 1996; Mah and O`Toole, 2001; Simões *et al.*, 2005a; Simões and Vieira, 2009). This is supposed to be due mainly to physiological characteristics of biofilm bacteria, but also to a barrier function of EPS (Morton *et al.*, 1998; Simões *et al.*, 2005a). The EPS matrix delays or prevents antimicrobials from reaching target microorganisms within the biofilm by diffusion limitation and/or chemical interaction with the extracelular proteins and polysaccharides (Heinzel, 1998; Mah and O`Toole, 2001). Moreover, within the EPS matrix the molecules required for cell-cell communication and community behaviour may accumulate at concentrations high enough to be effective (Sutherland, 2001a). The role of EPS components other than polysaccharides and proteins (fundamental structural elements of the biofilm matrix determining the mechanical stability of biofilms) remains to be established (Wingender *et al.*, 1999). Bacterial alginates represent an example of the few EPS which have been studied in detail, however, under the aspects of their relevance as a general virulence factor in infection processes of plants, animals, and man as well as in terms of their potential commercial exploitation (Wingender *et al.*, 1999). Lipids and nucleic acids might significantly influence the rheological properties and thus the stability of biofilms (Neu, 1996). The extracellular DNA is required for the initial establishment of biofilms by *P. aeruginosa*, and possibly for biofilms formed by other bacteria that specifically release DNA (Whitchurch *et al.*, 2002).

The signalling molecules produced by microorganisms involved on the process of cell-to-cell communication (quorum sensing) as described in section 2.4.4.

2.4 Mechanisms of biofilm formation in drinking water distribution systems

The understanding of the mechanisms of microbial growth in DWDS like the microbial ecology, specific mechanisms of adhesion, intra and interspecies interactions and the production of signalling and other metabolites molecules, will continue to provide needed insights to help resolve public health concerns associated with the biofilm formation on these systems. Since the standard methods of disinfection could not be efficient to control biofilms in DWDS. Recent findings into the microbial ecology of distribution systems have found that pathogenic resistance to chlorination is affected by microbial community diversity and interspecies relationships (Berry *et al.*, 2006).

2.4.1 Microbial community diversity

A DWDS provides a habitat for microorganisms, which are sustained by organic and inorganic nutrients present on the pipe and in the conveyed water (Payment and Robertson, 2004). According to Berry *et al.* (2006) an understanding of the microbial ecology of distribution system is necessary to design innovative and effective control strategies that will ensure safe and high quality DW to consumer.

In general, heterotrophic plate counts (HPC) are used to assess the overall bacterial quality of DW (Sartory, 2004). However, the majority of bacterial cells in natural communities are either non-cultivable by current cultivation methods or are present in a viable but non-cultivable (VBNC) state (Oliver, 2000). So, such methods are now known to significantly underestimate the total number of bacteria in DW (Szewzyk *et al.*, 2000). Thus, the real composition and dynamics of bacterial communities in DWDS are far from being assessed and understood in detail.

The biodiversity of bacterial population in DW biofilms is still poorly understood, but biomolecular tools bring recently new light on population composition and dynamics (Kalmbach *et al.*, 2000; Batté *et al.*, 2003; Martiny *et al.*, 2003, Schmeisser *et al.*, 2003; Williams and Braun-Howland, 2003, Williams *et al.*, 2004; Hoefel *et al.*, 2005; Eichler *et*

al., 2006). Through these molecular approaches, *Proteobacteria,* particularly of the classes *α-proteobacteria, β-proteobacteria, γ-proteobacteria and δ-proteobacteria,* have been found to predominate in chlorinated DW (Williams *et al.*, 2004; Eichler *et al.*, 2006; Poitelon *et al.*, 2009).

The microbial composition of DWDS communities is influenced by several factors and reflects the microflora characteristics of the raw water source (Eichler *et al*., 2006). Previous research has shown that distribution system pipe material, temperature, the level of organic carbon available, velocity of water and the disinfectant used in a system are among the factors that may impact the growth and community structure of a DWDS biofilms (Camper *et al.*, 1996; 2003; Percival *et al.*, 1999; Norton and LeChevallier, 2000, Williams *et al.*, 2004). According to Williams *et al.* (2004), following exposure to either of two disinfectants (free chlorine and monochloramine), *α-proteobacteria* was the predominant phylogenetic group observed in the treated distribution water, suggesting that these organisms are well suited to survive in potable water supplies. Whereas, *β-proteobacteria* were found to be more abundant in chloraminated water than in chlorinated water. In another study, Emtiazi *et al.* (2004) revealed that *β-proteobacteria* were also abundant in biofilms of non-chlorinated DW. These studies indicate that microbial community diversity is impacted by the disinfection strategy. There is also evidence that diversity can affect disinfection efficacy and pathogen survival (Berry *et al.*, 2006).

In DWDS, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Arthrobacter*/*Corynebacterium*, *Bacillus*, *Burkholderia*, *Citrobacter*, *Enterobacter*, *Flavobacterium*, *Klebsiella*, *Methylobacterium*, *Moraxella*, *Pseudomonas*, *Serratia*, *Staphylococcus*, *Mycobacterium*, *Sphingomonas* and *Xanthomonas* have been the predominant bacterial genera detected (Block *et al.*, 1997; Berry *et al.*, 2006). The Gram-negative are predominant over the Gram-positive bacteria, and *Pseudomonas* is the most abundant bacterial organism in supply systems, regardless the water source.

In a chloramianted sytems several authors detected ammonia- and nitrite-oxidazing bacteria such as, *Nitrosomonas* and *Nitrobacter* belonging to the *β-proteobacteria* and *α-proteobacteria,* respectively and the genus *Nitrospira* (Regan *et al.*, 2003; Hoefel *et al.*, 2005; Martiny *et al.*, 2005).

There are some published studies where no pathogens were detected in DWDS (Schmeisser *et al.*, 2003; Wingender and Flemming, 2004; Yu *et al.*, 2007). Most of the microorganisms developed in distribution network are harmless (Payment and Robertson,

2004). However, the dominant non-pathogenic bacterial populations should not be neglected, since they play a major role in biofilm formation (LeChevallier *et al.*, 1987) and biofouling (Bachmann and Edyvean, 2005). Nevertheless, other published studies detected several pathogens in DWDS such as: potentially pathogenic mycobacteria were detected in water samples collected in France (Le Dantec *et al.*, 2002); infectious enteroviruses and adenoviruses were detected in water samples in urban sites of Korea (Lee and Kim, 2002); opportunistic pathogens, *Mycobacterium* sp., *Legionella* spp. and *P. aeruginosa* were detected in biofilms and DW in Germany (Emtiazi *et al.*, 2004); *Helicobacter* spp. were identified in biofilms (Park *et al.*, 2001); *Aeromonas* spp. have also been found in DWDS (Sen and Rodgers, 2004), and in Russia and Bulgaria some water samples were positive for *Giardia* and *Cryptosporidium* (Karanis *et al.*, 2006).

Filamentous fungi and microfungi were also observed in DWDS (Doggett, 2000; Gonçalves *et al.*, 2006).

2.4.2 Coaggregation

Coaggregation, the specific recognition and adherence of genetically distinct bacteria to one another, occurs in a variety of ecosystems (Kolenbrander, 2000; Malik *et al.*, 2003; Rickard *et al.*, 2003a) and was first demonstrated for bacteria from dental plaque (Gibbons and Nygaard, 1970), where both intergeneric and intrageneric coaggregation occurs (Kolenbrander *et al.*, 1999). However, coaggregation is a widespread phenomenon has now been observed amongst bacteria from other biofilm communities in several diverse habitats. More recently, a few reports on the coaggregation abilities of freshwater biofilm bacteria have also been published (Buswell *et al.*, 1997; Rickard *et al.*, 2000; 2002; 2003a; 2004), and it has been suggested that coaggregation may also mediate in the sequential integration of species of bacteria into freshwater biofilms (Handley *et al.*, 2001; Rickard *et al.*, 1999). This mechanism of adhesion is highly specific and is thought to have a role in the development of multispecies biofilms in many different environments (Kolenbrander and London 1993; Kolenbrander *et al.*, 1999; Rickard *et al.*, 2003b) and now recognized as a mechanism for allowing specific association between collaborating bacteria species. Aggregation conveys advantages to microorganisms. These include transfer of chemical signals, exchange of genetic information, protection from adverse environmental conditions,

metabolic cooperation between different species, as well as cell differentiation in some populations (Wimpenny and Colasanti, 2004).

Coaggregation interactions contribute to the development of biofilms by two routes (Figure 2.8c). The first route is by single cells in suspension specifically recognizing and adhering to genetically distinct cells in the developing biofilm. The second is by the prior coaggregation in suspension of secondary colonizers followed by the subsequent adhesion of this coaggregate to the developing biofilm (Rickard *et al.*, 2003b). In both cases, bacterial cells in suspension specifically adhere to biofilm cells in a process known as coadhesion (Bos *et al.*, 1994; Busscher *et al.*, 1995).

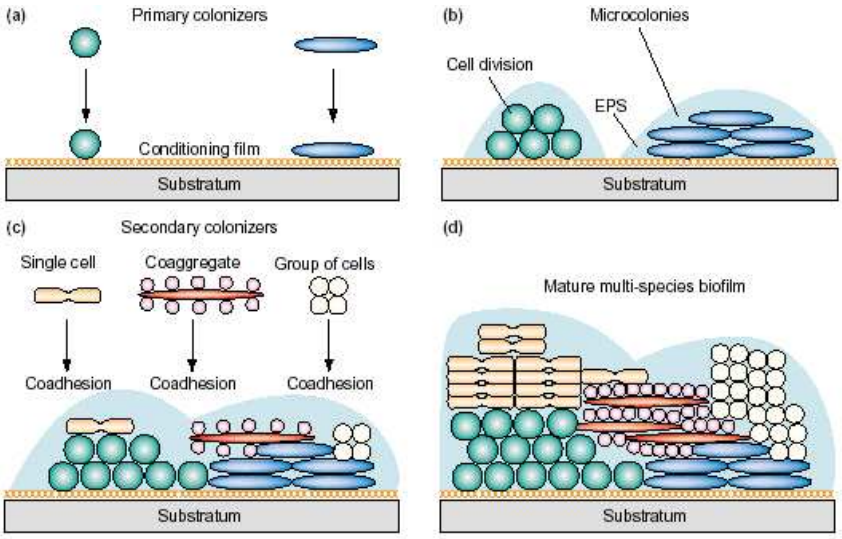


Figure 2.8 Illustration of the possible roles of coaggregation in the development of multispecies biofilms (adapted from Rickard *et al.*, 2003b). (a) Primary colonization of a substratum with a conditioning film; (b) cell growth, division and production of EPS leading to the development of microcolonies; (c) coadhesion of single cells, coaggregated cells and groups of identical cells into the young multispecies biofilm; and (d) maturation and formation of multispecies biofilm.

The coaggregation between pairs of freshwater bacteria is typically mediated by a protein "adhesin" on one cell type and a complementary saccharide "receptor" on the other. These protein-saccharide interactions could be blocked by the addiction of simple sugars. Thus, the mechanism mediating adhesion between coaggregating pairs in freshwater biofilm bacteria is very similar to the one verified by oral bacteria.

The coaggregation between freshwater bacteria is growth-phase-dependent. It depends on cells being in the optimum physiological state for coaggregation, being maximum when both partner bacteria are in stationary phase. Maximum expression of

coaggregation generates clearly visible flocs of cells in mixtures of the two types of cells (Rickard *et al.*, 1999) and is maintained for up to 48 h into stationary phase, depending on the coaggregating pair. The ability to coaggregate then decreases and eventually is lost completely (Rickard *et al.*, 2000). The optimum coaggregation between a pair might be dependent upon a change in coaggregation ability of one or both partner bacteria. As the adhesion on one bacteria and the receptor on the other partner bacteria may not be expressed simultaneously at all times in batch culture.

Studies on freshwater biofilm bacteria have also demonstrated that coaggregation often occurs between bacteria that are taxonomically distant (intergeneric coaggregation) and occasionally between strains belonging to the same species (intraspecies coaggregation) (Buswell *et al.*, 1997; Rickard *et al.*, 2002). Intergeneric coaggregation is common between oral bacteria (Kolenbrander and London, 1993), but intraspecies coaggregation has not yet been referred between oral plaque bacteria. Thus, intraspecies coaggregation may well be a characteristic that is unique to freshwater biofilm bacteria.

Moreover, and as suggested by Malik *et al.* (2003), the bacterial cell surface properties, namely the hydrophobicity, are other factor thought to play an important role in coaggregation, as well as in cell-substratum interactions.

In conclusion, bacteria are affected by the environment they live in and the variety of other species present. Coaggregation can take the form of intra, inter or multigeneric interactions, a combination of which contributes to the overall structure and diversity of bacterial community in the freshwater biofilms. The specific mechanism for this remains unknown, but a more complete picture of microbial community diversity and interspecies relationships should facilitate a better understanding of disinfection resistance phenomena and will provide new data to design innovative and effective control strategies that will guarantee microbial safe and high quality DW.

2.4.3 Microbial interactions

Under natural conditions, true monospecies biofilms are rare and in most natural and industrial environments, such as DWDS, biofilms are complex communities.

Diversity in microbial communities leads to a variety of complex relationships involving interspecies and intraspecies interactions. Interactions among bacterial species may have a profound influence on the initial stages of biofilm formation and development.

The ecology of a biofilm is a complex function of prevailing growth conditions, hydrodynamic forces, presence of microbial metabolites and molecules (cell-to-cell signalling communications) excreted by the microorganisms and dominant microbial inhabitants in the biofilm (Bryers and Ratner, 2004).

Surfaces provide a niche that promotes the evolution of complex interactions between bacterial cells. Once cells are firmly bound, the activity of the community is dependent on the metabolism and growth of each member species under local surface conditions. Such metabolic activities can include substrate consumption, cellular growth and replication, and synthesis of extracellular polymeric substances (Bryers and Ratner, 2004). The biological complexity of a system is defined by intra as well as interpopulation cell behaviour. The metabolic activities of those microorganisms that become associated with a surface cause these interfacial chemical gradients to evolve over time and space, creating conditions not normally encountered in the bulk aqueous phase (Geesey, 2001).

The microbial heterogeneity found in DW and the existence of interspecies relationships can provide improved strategies for microbial growth control (Rasmussen *et al.*, 2005). Competition for substrate is considered to be one of the major evolutionary driving forces in the microbial world, and experimental data obtained in laboratory conditions showed how different microorganisms may effectively outcompete others because of better utilization of a given energy source (Møller *et al.*, 1998; Christensen *et al.*, 2002). Central to the structure, composition and function of any community is a complex of interactions (Hansen *et al.*, 2007). For instance, Hansen *et al.* (2007) found that spatial structure was the key environmental factor for *P. putida* KT2440 and *Acinetobacter* sp. strain C6 to establish a structured community for interspecies interactions. Previously, Møller *et al.* (1998) showed the metabolic synergy between *P. putida* and *Acinetobacter* sp. community members when biodegrading toluene and related aromatic compounds. There is evidence that biofilm community diversity can affect disinfection efficacy and pathogen survival within biofilms (Burmølle *et al.*, 2006).

Most research into interspecies interactions within biofilms has focused on the beneficial aspects of these relationships. However, not all interactions will be beneficial, since antagonistic interactions may play an important role in the development of microbial communities. The production of antimicrobial molecules, including toxins, bacteriolytic enzymes, bacteriophages, antibiotics and bacteriocins seems to be a generic phenomenon for

most bacteria (Riley, 1998; Tait and Sutherland, 2002). Table 2.10 shows relevant interactions found for several multispecies biofilms from diverse environments.

Table 2.10 Relevant interspecies interactions in biofilm communities

Interspecies interactions	Strains	Reference
Antagonism	Marine epiphytic bacteria	Burgess *et al.* (1999)
	Enteric bacteria	Tait and Sutherland (2002)
Commensalism	*Acinetobacter* sp./*Pseudomonas putida*	Christensen *et al.* (2002)
	Lactococcus lactis ssp. *cremoris*/*Pseudomonas fluorescens*	Kives *et al.* (2005)
Competition	*Acinetobacter* sp./*Pseudomonas putida*	Christensen *et al.* (2002)
	Klebsiella oxytoca/*Burkolderia cepacia*	Komlos *et al.* (2005)
Mutualism (protocooperation and symbiose)	Soil bacteria	Wolfaardt *et al.* (1994)
	Oral bacteria	Palmer *et al.* (2001)
	Marine epiphytic bacteria	Burmølle *et al.* (2006)
Neutralism	*Pseudomonas* sp.; *Corynebacterium* sp.; *Candida* sp.; *Schizosaccharomyces* sp.; *Saccharomyces* sp.; *Schizosaccharomyces* sp.	Yu *et al.* (2002)

2.4.4 Cell-to-cell communication (quorum sensing)

The driving force in bacterial community development is the self-organization and cooperation among cells, rather than the classical "competitive" natural selection of individual microorganisms (Davies *et al.*, 1998; Fuqua and Greenberg, 2002; Daniels *et al.*, 2004; Parsek and Greenberg, 2005). This concept becomes particularly apparent when examining bacterial biofilm communities (Surette *et al.*, 1999; Parsek and Greenberg, 2005). Cell-to-cell signalling has been demonstrated to play a role in cell attachment and detachment from biofilms (Donlan, 2002; Daniels *et al.*, 2004).

Bacteria have the ability to signal and sense the state of population density in order to changing physiological needs under different growth conditions. This phenomenon is commonly called QS (Swift *et al.*, 1996). Therefore, QS is a strategy of cell-to-cell

communication benefiting the biofilm community by controlling unnecessary overpopulation and competition for nutrients (Davey and O'Toole, 2000). Bacteria are considered to be far from solitary microorganisms, and in fact are colonial by nature and exploit elaborate systems of intercellular interactions and communications to facilitate their adaptation to changing environments (Davies *et al.*, 1998; Sauer and Camper, 2001; Fuqua and Greenberg, 2002). The successful adaptation of bacteria to changing natural conditions is dependent on their ability to sense and respond to the external environment and modulate gene expression accordingly (Daniels *et al.*, 2004). QS is based on the process of auto-induction (Eberhard e*t al.*, 1981). The process of QS provides a mechanism for self-organization and regulation of microbial cells (Parsek and Greenberg, 2005). It involves an environmental sensing system that allows bacteria to monitor and respond to their own population densities. The bacteria produce a diffusible organic signal, originally called an auto-inducer (AI) molecule, which accumulates in the surrounding environment during growth (Fuqua and Greenberg, 2002). High cell densities result in high concentrations of signal, and induce expression of certain genes and/or physiological changes in neighbouring cells (Fuqua *et al.*, 1996; Parsek and Greenberg, 2005). A response to chemical signals in the process of cell communication is a concentration dependent process, where a critical threshold concentration of the signal molecule must be reached before a physiological response is elicited (Decho, 1999; Fuqua and Greenberg, 2002). Oligopeptides and *N*-acyl-homoserine lactones (AHL) are major AI molecules involved in intra-specific communication in Gram-positive and Gram-negative bacteria, respectively, whereas boronated diester molecules (AI-2) are involved in inter-specific communication among both Gram-positive and Gram-negative bacteria (Eberhard *et al.*, 1981; Fuqua and Greenberg, 2002; Parsek and Greenberg, 2005). AHL (AI-1) are the best characterized molecules (Eberhard *et al.*, 1981; Ryan and Dow, 2008).

QS systems are known to be involved in a range of important microbial activities. These include extracellular enzyme biosynthesis, biofilm development, antibiotic biosynthesis, biosurfactant production, EPS synthesis and extracellular virulence factors in Gram-negative bacteria (Passador *et al.*, 1993; Beck von Bodman and Farrand, 1995; Pearson *et al.*, 1995; Davies *et al.*, 1998; Daniels *et al.*, 2004; Fux *et al.*, 2005).

2.5 Biofilm control in drinking water distribution systems

Biofilm formation can be affected by several factors that can be manipulated in order to control their growth, but their elimination from DWDS is almost impossible. However, biofilm formation can be limited by:

- minimizing the concentration of organic matter entering the distribution system;
- ensuring the material from which the pipework and fittings are made are both chemically and biologically stable;
- maintenance of a disinfection residual through the distribution system;
- prevention of water stagnation and sediment accumulation within distribution systems (Gray, 2008).

However, in DWDS the main strategy to control the biofilm accumulation is the chemical disinfection by the increase of concentration of residual disinfectant through the network. However, this has to be done carefully, since the use of high chlorine concentrations cause aesthetic problems in the water (strong odour and tastes), increase the production of carcinogenic disinfection by-products, namely trihalomethanes and haloacetic acids, that are harmful to human health (Bull *et al.*, 1995; Nieuwenhuijsen *et al.*, 2000), and leads to the selection of resistant microorganisms. Currently, the residual concentration of free chlorine leaving the treatment plant is less than 1.0 mg l^{-1} and usually nearer to 0.5 mg l^{-1} (Gray, 2008). According to Zhou *et al.* (2009) the disinfectants levels usually employed in DWDS were not sufficient to prevent the growth and development of microbial biofilms. Consequently, the addition of supplementary chlorine in strategic points along the distribution system (re-chlorination stations) in order to maintain the disinfectant residual must be one further strategy to fight the chlorine decay in the distribution system and guarantee microbial content control in water.

On the other hand, when the water supplies have a high chlorine demand due to the presence of organic matter and humic acids, then it is difficult to maintain sufficient residual chlorine in the system. So, another strategy of control is to reduce the content of organic matter and nutrients in water by more effective pre-treatment. In general, the microorganisms need a C:N:P (carbon, nitrogen and phosphorous) ratio of 100:10:1 where

the carbon is the growth limiting nutrient, thus restricting the carbon concentration will decrease the microbial growth (Chandy and Angles, 2001; Payment *et al.*, 2003). However, the decrease of organic content, would be a very expensive process and ineffective for bacteria in DWDS which are able to grow in oligotrophic environments (Srinivasan and Harringtona, 2007; Zhou *et al.*, 2009). Also, this process cannot be used alone to preclude bacterial growth in the distribution system (Prévost *et al*., 1998; Flemming, 2002). Ultra-pure water systems have been found to support the formation of biofilms, even if these systems have lower organic content than the DWDS (Griebe and Flemming, 1998).

Other preventive strategies attempted to identify materials that do not promote or can even suppress biofilm formation (Rogers *et al.*, 1994). This study ranked different materials according to their biofilm growth propensity concluding that there is hardly any material that does not allow biofilm formation (Rogers *et al.*, 1994). However, there are studies which observed a considerable inhibitory effect of copper when compared to biofilm growth on other materials (high density PE, PVC, silicon, stainless steel and glass) (Mueller *et al.*, 1992; Schwartz *et al.*, 1998; Hem and Skjevrak, 2002). A previous study (West *et al.*, 1989) has already shown that copper was inhibitory to the colonization and growth of *L. pneumophila*. Copper ions were found to cause inhibition of the respiratory chain of bacteria (Domek *et al.*, 1984), which may contribute to low biofilm formation.

The type and stability of the material used in DWDS is another important factor that influences the proliferation of the biofilm in distribution system. It has been found that some pipes frequently experience problems with coliforms, taste and odor complaints. Also, there is a distinct development rate and microbial community structure of biofilms in different types of pipes (Lehtola *et al*., 2005). This was attributed to the leaching of nutrients for bacterial growth from the materials (Rogers *et al.*, 1994). Iron pipes support 10 to 45 times more growth than plastic pipes (Niquette *et al.*, 2000) and on other hand are more susceptible to residual disinfectant attack (Kerr *et al.*, 2003). The type of material also affects the disinfectant efficiency on the biofilm. Biofilms grown on copper, PE, PVC and cement-lined ductile iron were inactivated with a much lower amount of free chlorine or monochloramine than those grown on unlined iron surfaces (LeChevallier *et al.*, 1990; Norton and LeChevallier, 2000; Hallam *et al.*, 2002). This was explained by the interaction of chlorine with the iron, thus reducing the disinfectant residual. In cement-lined ductile iron, the cement provides a layer of protection for the iron against attack by chlorine. According to Al-Jasser (2007), the pipe service age is another important factor in the

consumption of chlorine and this effect decreases in the following order: cast iron > steel > cement-lined cast iron = cement-lined ductile iron > PVC = PE.

The distribution network must be planned to avoid zones of water stagnation or high water residence times in pipes. Pipes with long water residence times and dead-ends are associated with zones of high material organic settlement and consequently abundant biofilm formation. However, water stagnation occurs in every distribution system when DW consumption is low. Several reports associated higher bacterial numbers after periods of non-flow or storage of water in household pipes or tanks (LeChevallier *et al.*, 1987; Momba and Kaleni, 2002; Ayoub and Malaeb, 2006).

To attempt biofilm control in real DWDS the several strategies referred above should be used simultaneously. However, the potential formation of carcinogenic chemical byproducts has been reported as a drawback of traditional chemical disinfection methods. Therefore, there is a need to seek and develop new alternative techniques for water disinfection that would minimize environmental and public health impacts of traditional techniques.

Some authors reported other new and alternatives techniques for DW disinfection such as: UV irradiation and direct electrolysis, the combination of the two methods provides a promising approach to disinfection of DW and offer an attractive alternative to conventional methods (Bergmann *et al.*, 2002); water disinfection by acoustic and hydrodynamic cavitation, cavitation is a phenomena of formation, growth and collapse of microbubbles within a liquid, leads to the generation of very high pressures and temperatures locally, which can cause cellular damage (Jyoti and Pandit, 2001); water disinfection by the use of hybrid methods, simultaneous use of chemicals such as ozone and hydrogen peroxide with cavitation (Jyoti and Pandit, 2003). More recently, Li *et al.* (2008) reviewed the potential application of antimicrobial nanomaterials for water disinfection and microbial control. This study mentioned several natural and engineered nanomaterials (chitosan, silver nanoparticules, photocatalytic titanium dioxide, fullerol, aqueous fullerene nanoparticles and carbon nanotubes) that have been shown to have strong antimicrobial properties. Unlike conventional chemical disinfectants, these antimicrobial nanomaterials are not strong oxidants and are relatively inert in water. Therefore, they are not expected to produce harmful disinfection by-products. If properly incorporated into treatment processes, they have the potential to replace or enhance conventional disinfection methods. In other recent work, Shimizu *et al.* (2010) describes a disinfection method that utilizes ultrasound

irradiation of contaminated solution in the presence of titanium dioxide, a so-called "sonocatalytic disinfection" method. According to these authors, ultrasonic irradiation is well known as a useful technique for microbial inactivation due to its chemical and physical factors. Their recent studies indicated that the presence of titanium dioxide, known as a photocatalyst, accelerates the generation of hydroxyl radicals during ultrasonic irradiation, and that the process is mediated through the induction of cavitation bubbles in irradiating solutions. The significant role of hydroxyl radicals in the mechanism of cell-killing is also discussed in this work. More recently, Dankovich and Gray (2011) proposed a new water disinfection method that use a bactericidal paper impregnated with silver nanoparticles for point-of-use water treatment. This technique will be very useful for people that are not connected to DW network and for emergency situations following natural disasters.

Other recent approaches were studied for biofilm control, namely through the interference with some biological and ecological mechanisms involved in biofilm formation (microbial interactions and metabolite molecules). Several authors referred that the existence of multiple interspecies interactions or the simple production of a metabolite can interfere with biofilm formation and development (Tait and Sutherland, 2002; Carpentier and Chassing, 2004; Kives, *et al.*, 2005; Røssland *et al.*, 2005; Valle *et al.*, 2006). Some authors (Leriche and Carpentier, 2000; Zhao *et al.*, 2004) found that biofilm-forming microorganisms could play a role by interfering with the biological activities of pathogenic bacteria. Also, many bacteria are capable of synthesizing and excreting biosurfactants with anti-adhesive properties (Desai and Banat, 1997; Rodrigues *et al.*, 2004; van Hamme *et al.*, 2006; Nitschke and Costa, 2007). On the other hand the production of siderophores is a virulence factor in many microorganisms, acting as biocontrol molecules (Gram *et al.*, 1999). The discovery that many bacteria use QS to form biofilms makes it an attractive target for their control (Dunstall *et al.*, 2005; Rasmussen *et al.*, 2005). It is conceivable that QS inhibition may represent a natural, wide spread, antimicrobial strategy with significant impact on biofilm formation (Dong *et al.*, 2002). Recently, it was shown that AHL derivatives often not only have a function in modulating QS, but may also have direct bactericidal effects towards Gram-positive microorganisms like *Bacillus*, *Staphylococcus*, *Enterococcus* and others (Kaufmann *et al.*, 2005). Also farnesol, related to QS and biofilm formation in *Candida albicans* (Chen *et al.*, 2004), was reported to inhibit *S. aureus* biofilm formation and sensitize this organisms to antibiotics (Jabra-Rizk *et al.*, 2006).

Such biological and ecological mechanisms, alone or as part of synergistic procedures could provide a new line of efficient biofilm control strategies (Singh *et al.*, 2002; Banin *et al.*, 2005; Musk *et al.*, 2005).

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CHAPTER 3 BIOFILM FORMATION IN BIOREACTORS – ISOLATION OF DRINKING WATER BACTERIA

Monitoring of biofilm formation under different process conditions was performed using two distinct bioreactors, Propella™ and flow cell system. Biofilms were grown on PVC and stainless steel (SS) coupons under laminar (Reynolds number of 2000) and turbulent (Reynolds number of 11000) flow. The parameters analyzed were the numbers of cultivable cells, using R2A, and total bacteria, which were assessed using the DNA-binding stain 4,6-diamino-2-phenylindole (DAPI) coupled with epifluorescence microscopy. The impact of the different operating conditions in the studied parameters was established after the biofilms reached the steady-state. It was found that the biofilm steady-state was achieved 3 d after the starting of operating conditions for turbulent flow and for both bioreactors and adhesion surfaces. Under laminar flow it was only achieved 6 d after. The number of total bacteria was invariably higher than the cultivable cells. The numbers of total and cultivable bacteria in turbulent flow-generated biofilms were similar in both bioreactors, regardless the adhesion surface tested. Under laminar flow, the Propella™ bioreactor allowed the formation of steady-state biofilms with a higher number of total and cultivable bacteria than those from the flow cell system. Comparing the effects of the flow regime on biofilm accumulation, only turbulent flow-generated biofilms formed on the flow cell system had a higher amount of total and cultivable bacteria than those formed under laminar flow. In terms of adhesion surface effects on steady-state biofilms, a higher number of total and cultivable cells were found on PVC surfaces comparatively to SS when biofilms were formed using the flow cell system. Biofilm formation on PVC and SS was similar in the Propella™ system for both flow regimes. Diverse heterotrophic aerobic bacteria were isolated from these experiments enabling the study of relevant aspects on biofilm formation from DW bacteria and on their behaviour that are presented in the succeeding chapters.

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3.1 Introduction

The provision of microbiologically safe supplies of DW, following treatment, is one of the main goals that both DW companies and governments worldwide try to achieve and represents one of the cornerstones for maintenance of good public health (Szewzyk *et al.*, 2000; Deines *et al.*, 2010). However, the occurrence of waterborne diseases by ingestion of contaminated DW is still a major economic and, in some cases social burden, all around the globe. According to the WHO, diseases associated with unsafe water, sanitation and hygiene cause approximately 1.7 million deaths each year (Prentice, 2002). Disinfectant residuals, typically chlorine based, are normally used to reduce the numbers of microorganisms in DWDS. Nevertheless, increases in microbial numbers during distribution of DW have long been recognized (Baylis *et al.*, 1930), with microbial mediated processes contributing to the deterioration of water quality (Camper, 2004; Emtiazi *et al.*, 2004).

Biofilms are suspected to be the main source of microorganisms, including pathogens, in DWDS that are fed with treated water (LeChevallier *et al.*, 1987; Percival and Walker, 1999; Szewzyk *et al.*, 2000; Batté *et al.*, 2004; Codony *et al.*, 2005). The microorganisms in biofilms have a number of advantages over their counterparts, namely the production of extracellular polymeric matrix (capture and concentrate nutrients) that enables resistance to a number of control strategies (antimicrobial agents and shear stress conditions) (Simões *et al.*, 2005a; 2005b, 2007a). Although DWDS disinfection significantly reduces the numbers of planktonic bacteria, it has little to no effects on the numbers of biofilm bacteria (Gagnon *et al.*, 2005).

The dynamics of microbial growth in DW networks is very complex, as a large number of interacting processes are involved. Even though numerous environmental factors will influence biofilm formation in DWDS, including water temperature and pH, disinfectant type and residuals (Lund and Ormerod, 1995; Gagnon *et al.*, 2005), organic matter (Norton and LeChevallier, 2000), nutrient concentrations (Volk and LeChevallier, 1999; Chu *et al.*, 2005), surface material (Camper *et al.*, 1996), and hydraulics (Lehtola *et al.*, 2006), a complete understanding of how these factors act in concert to influence and control compositional changes during biofilm formation and detachment within DWDS remains a key challenge. The amount of biofilm in a given system after a certain period of time depends on biofilm accumulation, 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 that balance is null, the biofilm is said to have reached a steady-state. The final amount of biofilm in that state, which can be assessed by cell counts or biofilm mass, is directly related to the biofilm formation potential of that system (van der Kooij, 1999).

Research on DW biofilms has been performed in a wide variety of systems or biofilm monitoring bioreactor that should mimics the *in situ* situations with reproducible results; thus, important information is assessed about biofilm behaviour within the real DWDS. Several bench-top laboratory biofilm reactor systems, such as the rotating disc reactor (Murg *et al.*, 2001; Mhöle *et al.*, 2007), the CDC biofilm reactor (Goeres *et al.*, 2005), the biofilm annular reactor (Batté *et al.*, 2003a, 2003b), the Propella^{TM} reactor (Parent *et al.*, 1996; Appenzeller *et al.*, 2001), the Robbins device (Manz *et al.*, 1993; Kalmbach *et al.*, 1997), the modified Robbins device (McCoy *et al.*, 1981; Kharazmi *et al.*, 1999; Millar *et al.*, 2001); the flow cells systems (Simões *et al.*, 2006), the Prévost coupon (LeChevallier *et al.*, 1998; Prévost *et al.*, 1998); the Bioprobe monitor (LeChevallier *et al.*, 1998), the Pipe sliding coupon holder (Chang *et al.*, 2003), the biofilm sampler (Juhna *et al.*, 2007) and PWG coupon (Deines *et al.*, 2010) have been used to studying DW biofilms.

The complexity of the microenvironment under study and even the use of different methodologies and biofilm reactors systems lead in some cases to ambiguous or not easily comparable results. However, most studies assess only one variable at a time (e. g. Dunsmore *et al.*, 2002; Kerr *et al.*, 1999; Niquette *et al.* 2002; Pedersen, 1990; Rogers *et al.*, 1994a; Soini *et al.*, 2002; Zacheus *et al.*, 2000), and apart notable exceptions (Stoodley *et al.*, 1999; Block *et al.*, 1993; Simões *et al.*, 2006), scarce attempts have been made so far to study inter-relationships and compare the importance between these different factors.

The purpose of the present study was to evaluate biofilm formation by DW autochthonous bacteria on SS and PVC, two support materials commonly used on DW networks, under different water flow rates, using the Propella™ bioreactor and the flow cell system.

3.2 Material and methods

3.2.1 Bioreactors and biofilm monitoring

In this study, monitoring of DW biofilm subjected to different conditions was performed using two distinct bioreactors, flow cell system and Propella™. The configurations of these bioreactors are presented in Figure 3.1.

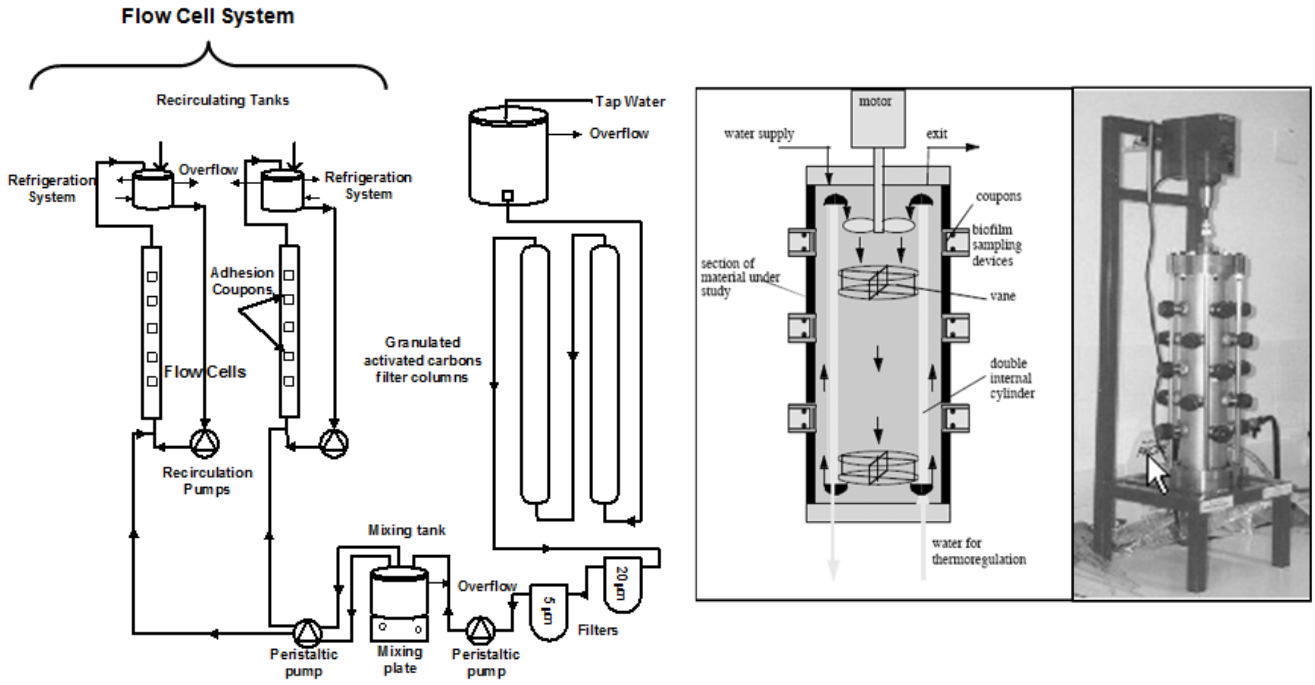

Figure 3.1 Experimental set-ups, showing the GAC filter columns and the flow cell system (a); and the Propella™ bioreactor (b).

Biofilms were grown on PVC and SS ASI 316 2R coupons. The water flow rate through the bioreactors was controlled by recirculating the water by means of recirculation pumps (flow cells) or by means of motor for water agitation (Propella™). The biofilms were developed under laminar (Reynolds number of 2000) and turbulent (Reynolds number of 11000) flow rates. Temperature in both bioreactors was maintained at 20 ± 1 ºC by an external refrigeration mechanism (Thermomix® BU, B. Braun – Biotech SA).

The biofilm experiment was carried on for at least 2 d after the biofilm reached a steady-state [considered to occur when constant values were obtained both for coloning forming units (CFU) and total bacterial cell counts (TB)], after which the experiment was terminated and the bioreactors sterilized.

3.2.1.1 Drinking water source

The DW source was from the public network in Braga (North of Portugal). Briefly, tap water was collected in a reservoir, which was connected to one of two consecutive granular activated carbons (GAC) filter columns. It has been shown elsewhere that the first GAC filter eliminates free chlorine and biodegradable matter contained in the tap water, while the second is a biological activated filter providing a continuous bacterial inoculum to the bioreactor (Morin and Camper, 1997). To avoid the presence of large carbon particles released from the columns, two filters (pore sizes 20 μm and 5 μm) were placed between the second GAC filter and the mixing tank. This tank supplied a constant inoculum at a flow rate of approximately 0.02 l h^{-1} into each of the flow cells or 1.12 l h^{-1} into the Propella™, in order to obtain the adequate dilution rate and similar to both bioreactor systems. Absence of free chlorine in the mixing tank was certified by regular sampling, using the free chlorine ion specific meter HI-93701 (Hanna Instruments, USA).

3.2.1.2 Flow cell system

The flow cell bioreactor is a pipe, with half-circle section, where adhesion coupons are placed on its inner flat surface. The flow cell may be directly connected to the tap and operates as a plug flow reactor or connected to a vessel that recirculates the water approaching a perfectly mixed reactor. This reactor system was designed to uncouple the system residence time and the fluid velocity by allowing water recirculation between the flow cell unit and a vessel. In the flow cell bioreactor several coupons, with the adhesion materials, are attached to the inner surface and may be replaced gradually without affecting the remaining system.

In this study, two flow cells were used in parallel, according to the procedure described by Pereira *et al.* (2002). Each one consists of a semicircular perspex duct with 43 cm in length and 1 cm of equivalent diameter (internal diameter of the half cylinder is 1.6 cm), where the biofilm coupons can be inserted. These rectangular coupons (2.4 cm length × 1.4 cm width), consisting of either SS or PVC, were glued to pieces of perspex that can be properly fitted in the apertures. Biofilms were formed on those coupons whose upper faces were in contact with the tap water circulating in flow cell reactor system. It was possible to remove separately each of the rectangular coupons without disturbing the biofilms formed on the others and without stopping the flow. This was managed because outlet ports were disposed on the round face of the flow cell between each two adjacent removable pieces of

perspex that allowed the deviation of the circulating flow from the point where the reactor was opened.

3.2.1.3 Propella™ bioreactor

The Propella^{TM} bioreactor is a perfectly mixed continuous reactor in which a propeller pushed the liquid down through the internal tube and up through the annular section between the two tubes. The flow rate inside the pipe was controlled by the rotation speed of the propeller and the residence time is proportional to the fresh inlet flow rate. In this reactor, the internal velocity and the hydraulic residence time may be chosen independently.

In this study, Propella^{TM} was made essentially of PVC and allowed to place 20 screwed biofilm sampling points in the inner reactor surface. On each sampling port, a circular coupon of SS and PVC surface material was glued. Biofilms were formed on those coupons whose upper faces were in contact with the tap water circulating in bioreactor.

3.2.2 Biofilm sampling

Biofilm sampling was made from the top to the bottom of the bioreactors under aseptic conditions and the coupons removed were substituted with new ones that were previously cleaned, immersed in ethanol (70% v v^{-1}) for 30 min, and rinsed in sterile distilled water. The removed coupons were gently washed with sterile sodium phosphate buffer (pH = 7.0) to remove loosely attached microorganisms and scraped with a scalpel into 15 ml glass tubes containing 10 ml of sterile phosphate buffer. Before serial dilutions, biofilm suspensions in the tubes were vortexed for 2 min and used to assess both CFUs and TB.

3.2.3 Cultivable and total cell counts

CFUs were evaluated by standard culture methods on R2A (Oxoid, UK) prepared according to the manufacturers instructions. Triplicate plates were used for each dilution and for each tested biofilm. CFUs were counted after 15 d of incubation at 20 ± 3 ºC, and the results were expressed as CFU cm^{-2}. TB were obtained by filtering the adequate volume (up to 10 ml as a function of the bacterial concentration) through a 25 mm black

Nucleopore® polycarbonate membrane with a pore size of 0.2 µm (Whatman, UK). Before the filtration step, 2% (v v^{-1}) formaldehyde (Merck, Germany) was added to the solution for sample fixation and preservation. After filtration, cells in the membrane were stained with 100 µg ml^{-1} of DAPI (Sigma, Portugal) for 5 min and the preparations were stored at 4 °C for up to 7 d in the dark, before visualization. No significant decay of fluorescence was noticed during this time span. Cells were visualised under an epifluorescence microscope (Carl Zeiss, Germany) equipped with a filter sensitive to DAPI fluorescence (359 nm excitation filter in combination with a 461 nm emission filter). A total of 20 fields were counted and the average of three membranes was used to calculate total cells per cm^2.

3.2.4 Statistical analysis

Paired t-test analyses were performed to estimate whether or not there was a significant difference between the results obtained. Statistical calculations were based on a confidence level equal or higher than 95% (a *P* value < 0.05 was considered statistically significant).

3.3 Results and Discussion

The examination of a DWDS reveals the complexity of such a technical system. There are not only many different materials used for the transportation and regulation of the water flow but also dramatic variations in the flow conditions between different locations. Obviously, microorganisms face a diversity of habitats with distinct physicochemical and nutritional conditions during treatment, storage, and distribution of DW.

Biofilms constitute a protected mode of growth that allows microorganisms to survival in hostile conditions, being their phenotype significantly different from their planktonic counterparts. Their development, behaviour and population characteristics are strongly influenced by many environmental factors and by intrinsic biological properties (Sauer and Camper, 2001; Purevdorj *et al.*, 2002). From the most important environmental factors affecting biofilm structure and behaviour are the velocity field of the fluid in contact with the microbial layer and the support material for bacterial adhesion and further biofilm development (Vieira *et al.*, 1993; Stoodley *et al.*, 1999; Simões *et al.*, 2006). Hydrodynamic

conditions will determine the rate of transport of cells, oxygen and nutrients to the surface, as well as, the magnitude of shear forces acting on a developing biofilm (Vieira *et al.*, 1993). Regarding the effects of the support material, microbial attachment will occur most readily on surfaces that are rougher, more hydrophobic, and coated by surface conditioning films (Donlan, 2002).

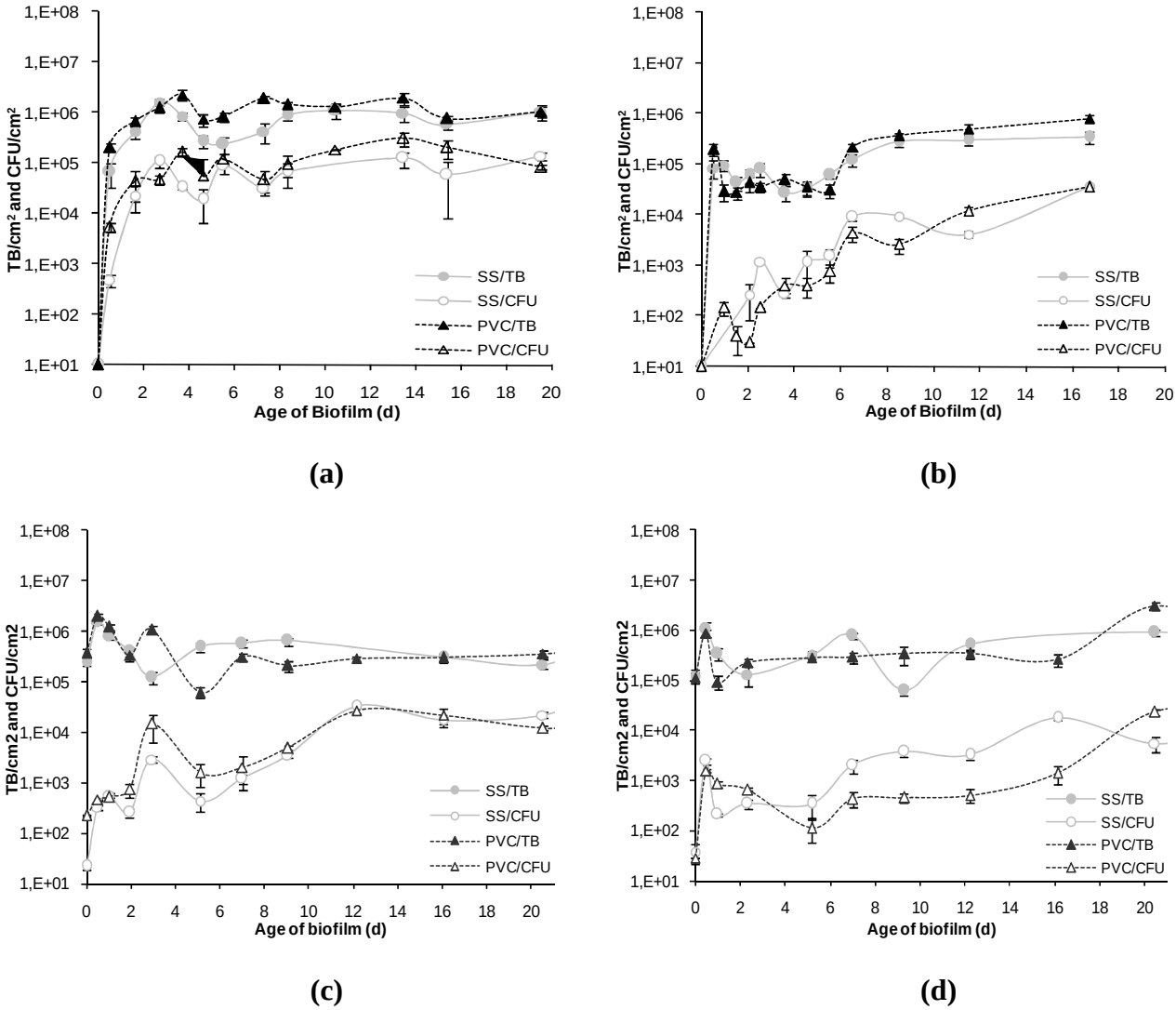

Figure 3.2 Kinetics of biofilm growth obtained for the different conditions with the two bioreactors. Biofilm accumulation along time (assessed by TB and CFU) on SS and PVC surfaces. (a) turbulent flow and (b) laminar flow in flow cell bioreactor. (c) turbulent flow and (d) laminar flow in the Propella^{TM} bioreactor.

Biofilm accumulation in all experiments, expressed both in CFU and TB, increased markedly in the first few days, following a sigmoidal curve (Figure 3.2). Biofilm steady-state was achieved 3 d after the starting of operating conditions for turbulent flow conditions

and for both bioreactors and adhesion surfaces. Under laminar flow conditions, it was only achieved 6 d after. For those cases, the number of total bacteria was invariably higher than the cultivable cells (differences always higher than 2 log). It has long been recognized that the use of culture-based enumeration techniques may significantly underestimate the numbers of viable cells. Several reasons may account for this difference: the presence of starved or injured cells or potentially VBNC cells that are not able to initiate cell division at a sufficient rate to form colonies; inadequate culture conditions; aggregation of bacteria that can lead to the formation of one colony from more than one cell, thereby underestimating the total number of cells (Banning *et al.,* 2002). However, total and cultivable bacteria in turbulent flow-generated biofilms were similar in both bioreactors, regardless the adhesion surface ($P > 0.05$). This result suggests that increased hydrodynamic stress favours biofilm bacteria cultivability. Vieira *et al.* (1993) found that mass transfer limitations existed in a higher extent in biofilms formed under laminar flow than for turbulent conditions. Consequently, the higher oxygen rate and transport of substrate, even if at very low levels in DWDS, from the fluid to the biofilm (mass transfer effects) should favour microbial metabolism and cell replication. Comparing the effects of the flow regime on biofilm accumulation, it was only found for the flow cell system that turbulent flow-generated biofilms had a higher amount of total and cultivable bacteria than those formed under laminar flow ($P < 0.05$). This result is in agreement with previous studies (Stoodley *et al.*, 1999; Simões *et al.,* 2007c), with single and mixed species biofilms formed on flow cell systems, showing that biofilms formed under turbulent flow had a significant higher cell density than the laminar counterparts. Turbulent and laminar flow-generated biofilms formed on the Propella™ bioreactor had comparable cell densities. Moreover, the Propella™ system allowed the formation of steady-state laminar flow-generated biofilms with a higher number of total and cultivable bacteria than those formed on the flow cell system ($P < 0.05$). In fact, there are significant differences on the design of the used bioreactor systems that can account for the differences obtained. For example, the hydrodynamic stress is obtained by distinct mechanisms when using Propella™ bioreactor (agitation by means of a rotating device system) and the flow cell system (fluid flow). In terms of adhesion surface effects, in the flow cell system, a higher number of cells formed biofilms on PVC surfaces comparatively to SS ($P < 0.05$), while biofilm formation on PVC and SS was similar ($P > 0.05$) in the Propella™ system, for both flow regimes. In a previous study (Simões *et al.*, 2007b), it was demonstrated that the tested materials had similar

physico-chemical characteristics, such as hydrophobicity, and both are prone to colonization by DW isolated bacteria. Consequently, taking into account the physico-chemical characteristics, it was expected low biofilm data variability as a consequence of adhesion surface differences.

These experiments enabled the isolation of heterotrophic aerobic bacteria that were characterized in more detail, in terms of key aspects promoting biofilm formation and resistance to control conditions, and these results are presented in the succeeding chapters.

3.4 Conclusions

The development and validation of reliable biofilm monitoring techniques is required in order to mimic real environmental situations using laboratorial systems. This work demonstrates that distinct bioreactor configurations provide different biofilm data. In fact, the use of PVC or SS as adhesion surfaces and distinct hydrodynamic conditions lead to biofilm accumulation variability in terms of CFU and TB when using the Propella™ or the flow cell bioreactors. Moreover, this study highlights the need for a deeper understanding of how the large spectrum of conditions interact and affect biofilm formation potential and accumulation with the final purpose of predicting the total and cultivable bacteria attached to real DW distribution pipes, based on the system characteristics. Although the practical use of these conclusions by DW network companies is still limited, the information provided here might be used as a framework for future studies.

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CHAPTER 4 CHARACTERIZATION OF DRINKING WATER BACTERIA, MATERIAL SURFACES AND STUDY OF ADHESION POTENTIAL

Heterotrophic bacteria (11 genera, 14 species, 25 putative strains) were isolated from DW, identified either biochemically or by partial 16s rDNA gene sequencing and their adherence characteristics were determined by two methods: i. thermodynamic prediction of adhesion potential by measuring hydrophobicity (contact angle measurements) and ii. by measuring adherence to eight different substrata (ASI 304 and 316 SS, copper, PVC, polypropylene (PP), PE, silicone and glass). All the test organisms were hydrophilic and inter-species variation in hydrophobicity occurred only for *Comamonas acidovorans.* Stainless steel 304 (SS 304), copper, PP, PE and silicone thermodynamically favoured adhesion for the majority of test strains (> 18/25), whilst adhesion was generally less thermodynamically favourable for stainless steel 316 (SS 316), PVC and glass. The predictability of thermodynamic adhesion test methods was validated by comparison with 24-well microtiter plate assays using nine reference strains and three adhesion surfaces (SS 316, PVC and PE). Results for *Acinetobacter calcoaceticus*, *Burkolderia cepacia* and *Stenotrophomonas maltophilia* sp. 2 were congruent between both methods whilst they differed for the other bacteria to at least one material. Only *A. calcoaceticus* had strongly adherent properties to the three tested surfaces. Strain variation in adhesion ability was detected only for *Sphingomonas capsulata*. Analysis of adhesion demonstrated that in addition to physicochemical surface properties of bacterium and substratum, biological factors are involved in early adhesion processes, suggesting that reliance on thermodynamic approaches alone may not accurately predict adhesion capacity.

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4.1 Introduction

Bacterial adhesion to surfaces is one of the initial steps leading to biofilm formation and is therefore an important microbiological event in medicine (Costerton *et al.*, 1987), industry (Simões *et al.*, 2005) and the environment (Bayoudh *et al.*, 2005; Simões *et al.*, 2006a). In DWDS, microbial adhesion will initiate biofilm formation, exacerbating contamination of DW, reducing the aesthetic quality of potable water, increasing the corrosion rate of pipes and reducing microbiological safety through increased survival of pathogens (Percival and Walker, 1999; Niquette *et al.*, 2000; Tsai, 2005; Simões *et al.*, 2006a). Microorganisms are generally less of a problem in planktonic phase since due to increased disinfection efficiency (Simões *et al.*, 2003, 2005, 2006b). Considerable resources have therefore been directed towards technologies designed to inhibit the microbial attachment with the aim of deriving colonisation-free surfaces (Thouvenin *et al.*, 2003, Tang *et al.*, 2005). Microbial adhesion to surfaces is a complex process, influenced by several physicochemical properties of both microorganism and substratum, the most significant of which are hydrophobicity and surface charge (Donlan, 2002; Gallardo-Moreno *et al.*, 2002a, 2002b). The initial adhesion step can be interpreted in terms of Lifshitz-van de Waals forces (LW) and acid-base forces (AB) (Smets *et al.*, 1999; Gallardo-Moreno *et al.*, 2002a). When a microorganism and a surface enter into direct contact the water film present between the interacting entities has to be removed. This is in accordance with the thermodynamic theory of adhesion and is expressed by the Dupré equation which states that the Gibbs free energy of interaction can be calculated assuming that the interfaces between bacteria/liquid medium and solid/liquid medium are replaced by a bacteria/solid interface (Absolom *et al.*, 1983). Accordingly, hydrophobicity has been considered the most important short-range interaction force in bacterial attachment, playing a determinant role in bacterial adhesion (van Oss, 1997). Other cellular and support material associated inherent factors can also significantly account for the adhesion process (Flint *et al.*, 1997; Sinde and Carballo, 2000). For example, the production of polysaccharides, lipopolyssacharide chemistry, and other factors may also affect adhesion (Li and Logan, 2004) even if their contribution is not incorporated into predictive models. The purpose of the present study was to characterize the physicochemical properties of surfaces of a selection of numerically important DW isolates and to evaluate their potential for adhesion to a variety of materials with potential use in DW distribution

pipes, comparing predicted adhesion based on thermodynamic approaches with adhesion assays.

4.2 Material and methods

4.2.1 Bacteria isolation and growth

The microorganisms used throughout this work were isolated from a model laboratory DWDS, as described in the previous chapter and by Simões *et al.* (2006a). Briefly, the system consisted of a Perspex vessel (volume, 1.6 l; diameter, 16.8 cm) fed with normal tap water in Braga, Portugal. The system was sterile until filled with potable water and operated so as to prevent immigration of microorganisms other than via the tap water feed. The flow rate of tap water gave a dilution rate of 3.125 h^{-1}. Microorganisms were isolated by collecting 100 µl chemostat water and plating on trypticase soy agar (TSA) (Merck, VWR, Portugal) and R2A (Oxoid, UK) aerobically at room temperature for 15 days. TSA and R2A were selected since in validation studies (data not shown) they supported the optimal growth, successfully recovering heterotrophic bacteria from DW. R2A has been previously validated as an effective isolation medium for aquatic bacteria (Reasoner and Geldrich, 1985; Simões *et al.*, 2006a).

4.2.2 Bacterial identification

Preliminary, presumptive bacteria identification was done using selective medium Chromocult® TBX (Tryptone Bile X-glucuronide) agar (Merck); *Pseudomonas* isolation agar (Difco); Metanol minimum medium, according to Kim *et al.* (1999), Gram-staining and biochemical methods (API 20 NE and API ID32 GN systems (Biomerieux)), according to the manufacturer's instructions. Further identification tests, by determination of 16S rDNA gene sequence, were performed for putative *Acinetobacter* sp., *Burkolderia* spp., *Methylobacterium* spp., *Pseudomonas* spp., *Sphingomonas* spp. and *Stenotrophomonas* spp. As follows: Genomic DNA was extracted and purified by applying an isolated colony, collected from pure plate cultures resuspended in 20 µl of TE buffer, in indicating FTA Classic Cards (WB120206, Whatman) and proceeding according to manufacturer's instructions. 16S rDNA was amplified with universal primers pA (5'- AGA GTT TGA TCC

TGG CTC AG) and pH (5'- AAG GAG GTG ATC CAG CCG CA-3') (Ulrike *et al.*, 1989) or 109F (5'- ACG GGT GMG TAACKC GT-3') and 1392R (5'- ACG GGC GGT GTG TRC-3') (Lane, 1991). PCR was performed in a Thermocycler (Uno II, Biometra) and the reaction (mixtures containing the template DNA in the FTA disc) occurred in 35 cycles with 1 min denaturation at 96 ºC, 1 min annealing at 50 ºC and 1 min extension at 72 ºC, after a previous step of denaturation (96 ºC for 4 min) and followed by a final extension step (72 ºC for 5 min). PCR products were visualized using ethidium bromide staining after electrophoresis through a 1% (w v^{-1}) agarose gel and their sizes were determined by comparison with a molecular weight standard (1kb plus DNA ladder; GibcoBRL). The PCR products were purified using Jet Quick-PCR Purification Kit (Genomed, Germany) as described by the manufacturer. Sequecing was done using an automated DNA capillary sequencer CEQ 2000-XL (Beckman Coulter, USA, in ICAT-Lisbon Faculty of Sciences Sequencing Services) by a dye-labeled dideoxy termination method (DTCS, Dye Terminator Cycle sequencer start kit, Beckman Coulter). Five sequencing reactions were performed using the two primers for PCR amplification and internal primers 534R (5'- ATT ACC GCG GCT GCT GG-3'), 907R (5'- CCG TCA ATT CMT TTR AGT TT-3') and 926F (5'-AAA CTY AAA KGA ATT GAC GG-3') (Lane, 1991). For each strain, the partial 16S rDNA sequence was assembled by combining the sequences generated by each primer, using the CEQ Investigator program (software CEQ 8000, Beckman Coulter). The sequences were compared with National Centre for Biotechnology Information GenBank entries using BLAST algorithm (Altschul *et al.*, 1990).

4.2.3 Adhesion substrata

The materials assayed were ASI 304 stainless steel (SS 304), ASI 316 stainless steel (SS 316), copper, glass, PVC, PP, PE and silicone (Neves & Neves, Muro, Portugal). Some of these materials (SS 304, SS 316, PVC, PP, PE, copper) are commonly used in DW distribution networks, while other materials were used for comparative purposes (glass, silicone). In order to prepare the materials for further analysis, they were immersed in a solution of commercial detergent (Sonasol Pril, Henkel Ibérica S. A.) and ultrapure water for 30 min. In order to remove any remaining detergent, the materials were rinsed in ultrapure water and subsequently immersed in ethanol at 96% (v v^{-1}) for 30 min, except for PVC, PP, PE and silicone that were immersed for 10 s. After being rinsed three times with

ultrapure water, the materials were dried at 65 ºC for 3 h before being used in contact angle measurements and for adhesion assays.

4.2.4 Bacterial cell growth and preparation

Bacterial cells were grown in batch culture using TSB medium (Merck, VWR, Portugal), at room temperature (23 ºC ± 2), under agitation (150 rpm), until reaching the stationary phase of growth as assessed by spectrometry. Cells were harvested by centrifugation (10 min at 7000 rpm), washed three times in phosphate buffer saline (PBS) (0.1 M, pH 7.2) and resuspended in PBS (200 ml ± 10) in order to achieve the bacterial concentration required for each assay.

4.2.5 Surface contact angle measurements

Bacterial lawns for contact angle measurements were prepared as described by Busscher *et al.* (1984). The surface tension of the bacterial surface and of the tested materials were then determined using the sessile drop contact angle method. The measurements were carried out at room temperature (23 ºC ± 2) using three different liquids: water, formamide and α-bromonaphtalene (Sigma, Portugal). 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 25 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).

4.2.6 Surface hydrophobicity and free energy of adhesion

Hydrophobicity was assessed after contact angle measurements and using the approach of van Oss *et al.* (1987, 1988, 1989). In this approach, the degree of hydrophobicity of a given material (1) is expressed as the free energy of interaction between two entities of that material when immersed in water (w) - ΔG_{1w1}. If the interaction between the two entities is stronger than the interaction of each entity with water $\Delta G_{1w1} < 0$

the material is considered hydrophobic. Conversely, if $\Delta G_{1w1} > 0$ the material is hydrophilic. ΔG_{1w1} can be calculated through the surface tension components of the interacting entities, according to:

$$\Delta G_{1w1} = -2\left(\sqrt{\gamma_1^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_1^+\gamma_w^-} + \sqrt{\gamma_1^-\gamma_w^+} - \sqrt{\gamma_1^+\gamma_1^-} - \sqrt{\gamma_w^+\gamma_w^-}\right) \quad (1)$$

where γ^{LW} accounts for the Lifshitz-van der Waals component of the surface free energy and γ^+ and γ^- are the electron acceptor and electron donor parameters, respectively, of the Lewis acid-base component (γ^{AB}), with $\gamma^{AB} = 2\times\sqrt{\gamma^+\gamma^-}$.

The surface tension components of a solid material are obtained by measuring the contact angles of three pure liquids (one apolar - α-bromonaphtalene and two polar – water and formamide), with well known surface tension components, followed by the simultaneous resolution of three equations of the form:

$$(1+\cos\theta)\gamma_1^{TOT} = 2\left(\sqrt{\gamma_s^{LW}\gamma_1^{LW}} + \sqrt{\gamma_s^+\gamma_1^-} + \sqrt{\gamma_s^-\gamma_1^+}\right) \quad (2)$$

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

When studying the interaction between substances 1 and 2 that are immersed or dissolved in water (w), the total interaction energy, ΔG_{1w2}^{TOT}, can be expressed as:

$$\Delta G_{1w2}^{TOT} = \gamma_{12}^{LW} - \gamma_{1w}^{LW} - \gamma_{2w}^{LW} + 2\left[\sqrt{\gamma_w^+}\left(\sqrt{\gamma_1^-} + \sqrt{\gamma_2^-} - \sqrt{\gamma_w^-}\right) + \sqrt{\gamma_w^-}\left(\sqrt{\gamma_1^+} + \sqrt{\gamma_2^+} - \sqrt{\gamma_w^+}\right) - \sqrt{\gamma_1^+\gamma_2^-} - \sqrt{\gamma_1^-\gamma_2^+}\right] \quad (3)$$

Thermodynamically, if $\Delta G_{1w2}^{TOT} < 0$, adhesion is favourable. On the contrary, adhesion is not expected to occur if $\Delta G_{1w2}^{TOT} > 0$.

4.2.7 Adhesion assays

Adhesion assays were performed with 9 representative bacteria, respectively, *A. calcoaceticus, B. cepacia, Methylobacterium sp., M. mucogenicum, Sph. capsulata* sp. 1 and sp. 2, *Staphylococcus sp.,* and *S. maltophilia* sp. 1 and sp. 2 using PE, PVC and SS 316 as representative adhesion surfaces. Coupons of materials with 8 mm × 8 mm, prepared as indicated previously, were inserted in the bottom of 24-wells (15 mm diameter each well) microtiter plates (polystyrene, Orange Scientific, USA) and 2 ml of each cell suspension (10^9 cells ml^{-1} in PBS), was added to each well. Adhesion to each material was allowed to

occur for 2 h at room temperature, in a shaker at 150 rpm, according to the methods of Cerca (2006). Negative controls were obtained by placing materials in PBS without bacterial cells. The experiments were performed in triplicate and repeated three times. At the end of the assay each well was washed twice with PBS, by pipetting carefully only the liquid above the coupon. After the last wash, the coupons were removed from each well and immersed in a new microtiter plate containing 1 ml of methanol 98% (v v^{-1}) in each well (Henriques *et al.*, 2005). Methanol was withdrawn after 15 min of contact and the coupons were allowed to dry at room temperature. Aliquots (600 µl) of crystal violet (CV) were then added to each well and incubated for 5 min. After gently washing in water the coupons were left to dry, before being immersed in 1 ml of acetic acid 33% (v v^{-1}) to release and dissolve the stain. The optical density (OD) of the obtained solution was measured at 570 nm using a microtiter plate reader (BIO-TEK, Model Synergy HT).

Bacteria were classified using the scheme of Stepanović *et al.* (2000) as follows: Non-adherent (0): $OD \leq OD_c$; weakly adherent (+): $OD_c < OD \leq 2 \times OD_c$; moderately adherent (++): $2 \times OD_c < OD \leq 4 \times OD_c$; strongly adherent (+++): $4 \times OD_c < OD$. This classification was based upon the cut-off OD (OD_c) value defined as three standard deviation values above the mean OD of the negative control.

4.2.8 Statistical analysis

The data were analysed using the statistical program SPSS version 14.0 (Statistical Package for the Social Sciences). Because low samples numbers contributed to uneven variation, the adhesion results were analyzed by the nonparametric Wilcoxon test. Statistical calculations were based on a confidence level of ≥ 95% ($P < 0.05$ was considered statistically significant).

4.3 Results and discussion

4.3.1 Characterisation of DW bacteria

In this study, 25 phenotypically distinct autochthonous DW bacteria were isolated (Table 4.1), belonging to 14 different bacterial species. Several of the isolates (*Acinetobacter* spp., *Burkholderia* spp., *Comamonas* spp., *Methylobacterium* spp.,

Mycobacterium spp., *Pseudomonas* spp., *Sphingomonas* spp., *Stenotrophomonas* spp.) have previously been detected in DW (Kuhn *et al.*, 1997; Norton and LeChevallier, 2000; Zanetti *et al.*, 2000; Rickard *et al.*, 2004; Stelma Jr. *et al.*, 2004). The test organisms were analyzed in terms of hydrophobic surface characteristics.

Table 4.1 Values of contact angle (in degrees) with water (θ_W), formamide (θ_F), α-bromonaphtalene (θ_B), surface tension parameters and free energy of interaction (ΔG_{bwb}^{TOT}) of the isolated microorganisms (b) when immersed in water (w). Values are means ± SDs

Bacteria		Contact angle (°)			Surface tension parameters (mJ m^{-2})			Hydrophobicity (mJ m^{-2})
		θ_W	θ_F	θ_B	γ_b^{LW}	γ_b^+	γ_b^-	ΔG_{bwb}^{TOT}
Acinetobacter calcoaceticus		50.9±2.6	45.5±3.5	64.4±2.4	22.8	3.1	31.2	7.0
Burkholderia sp.		35.8±2.1	38.0±1.5	66.6±1.5	21.7	4.0	45.6	20.8
Burkholderia cepacia		22.8±1.3	37.2±4.5	54.2±3.0	27.9	1.4	60.9	42.0
CDC gr. IV C-2	sp. 1	19.2±1.6	39.2±3.3	51.9±4.7	29.0	0.8	66.6	51.0
	sp. 2	21.8±3.2	34.8±4.1	56.7±2.4	26.7	2.0	59.5	38.2
Comamonas acidovorans	sp. 1	52.0±3.6	100.2±8.2	51.2±2.6	29.4	0.0	117.1	115.4
	sp. 2	52.5±4.4	57.8±4.7	39.2±2.6	35.0	0.0	42.1	26.0
	sp. 3	40.0±2.0	50.0±2.1	75.5±2.0	17.4	3.0	51.2	27.5
	sp. 4	28.2±2.3	38.9±2.0	57.9±1.4	26.0	1.8	56.5	36.4
Methylobacterium sp.		14.0±3.6	25.9±3.5	47.0±3.3	31.4	1.8	58.9	37.1
Methylobacterium mesophilicum	sp. 1	23.1±3.3	30.3±3.2	34.6±4.5	36.9	0.6	55.6	37.3
	sp. 2	26.6±3.4	36.9±3.7	46.3±2.1	31.7	0.8	57.1	39.8
	sp. 3	16.8±3.1	24.5±2.9	48.4±2.3	30.7	2.3	56.0	32.9
	sp. 4	35.0±2.3	44.3±3.3	54.9±2.4	27.5	0.9	53.7	36.7
	sp. 5	16.8±1.9	27.8±1.7	48.4±2.2	30.7	1.8	58.3	36.7
	sp. 6	30.7±2.5	33.4±1.5	55.8±2.3	27.1	2.6	48.8	26.0
Moraxella lacunata		41.9±2.5	44.7±2.4	78.3±1.9	16.1	5.5	42.6	15.2
Mycobacterium mucogenicum		37.4±3.2	71.4±2.9	62.5±3.6	23.7	0.0	89.4	88.9
Pseudomonas sp.		56.0±2.0	63.8±2.5	88.4±2.1	11.7	3.0	40.4	14.3
Pseudomonas reactans		28.0±2.1	36.2±2.4	63.3±2.9	23.3	3.1	53.9	30.0
Sphingomonas capsulata	sp. 1	47.7±2.6	47.1±4.5	51.3±7.9	29.3	0.8	38.0	17.5
	sp. 2	40.4±1.9	46.5±1.7	86.9±1.7	12.3	7.1	46.0	13.8
Staphylococcus sp.		24.9±1.9	22.9±2.9	78.6±1.4	15.9	10.6	46.2	11.6
Stenotrophomonas maltophilia	sp. 1	48.8±1.8	47.0±2.8	53.6±3.2	28.2	1.1	36.2	14.7
	sp. 2	32.8±3.0	32.0±1.6	72.0±2.1	19.0	6.8	44.1	15.4

All the bacteria had a water contact angle lower than 65º and a $\Delta G_{bwb}^{TOT} > 0$ (Table 4.1), fitting the hydrophilic classification. According to Vogler (1998), surfaces that have a contact angle higher than 65º are classified as hydrophobic; conversely, hydrophilic surfaces are the ones with water contact angle values lower than 65º. However, based on the water contact angle approach, hydrophobicity can only be qualitatively analyzed (Oliveira *et al.*, 2001). The application of the van Oss (1997) approach, which allows the assessment of the absolute degree of hydrophobicity of any substance in comparison with water as a quantitative result, seems to be more accurate than the assessment of the water contact angles, since it comprises the contact angle values of three different liquids (water, formamide and α-bromonaphtalene).

Comparisons of the surface characteristics of bacteria within the same species, detected the existence of significant inter-strain differences in water contact angles and ΔG_{bwb}^{TOT} values, although these were variations in the extent of hydrophilic properties rather than between hydrophilic and hydrophobic. These differences are more pronounced for the *Comamonas acidovorans* strains, where ΔG_{bwb}^{TOT} ranged from 26 to 115 mJ m^{-2}. van der Mei *et al.* (1998), Teixeira *et al.* (2005) and Chae *et al.* (2006) also observed a large variation in the degree of hydrophobicity among strains of the same species, emphasizing that generalization concerning the surface properties of bacterial cells, based on their identity should be made with caution. The water contact angle measurements did not allow the grouping of strains according to their taxonomy as reported previously by van der Mei *et al.* (1998) and Teixeira *et al.* (2005).

Comparing the results obtained with those reported in the literature, *A. calcoaceticus* gave water contact angle values in agreement with those reported by van der Mei *et al.* (1998). Concerning the other test organisms, no previous reports were found concerning cell surface hydrophobicity characterization.

C. acidovorans sp. 1, sp. 2, *M. mucogenicum* and *Pseudomonas* sp., had formamide contact angles above 55º, meaning that the polar characteristics of the bacteria were different from the other tested bacteria. These bacteria, with exception of the *Pseudomonas* sp., were only electron donors and the acid base component of the surface free energy approaches zero (Bos *et al.*, 1999). It is not surprising that cell surface properties of *M. mucogenicum* were considerably different from the other bacteria due to the presence of a waxy cell wall. The differences found between the *C. acidovorans* strains may be related to

the different surface properties of the bacterial cell wall such as the ability to produce different exopolymeric substances (Li and Logan, 2004) and surface proteins (Schär-Zammaretti *et al.*, 2005).

The Lifshitz-van der Waals surface tension component of the microorganisms was comprised between 11.7 mJ m^{-2} and 36.9 mJ m^{-2}, the lowest value being that of *Pseudomonas* sp. and the highest the one for *M. mesophilicum* sp.1. All the bacteria were predominantly electron donors (electron-donating component between 31.2 and 117.1 mJ m^{-2}) and, except *C. acidovorans* sp. 1, sp. 2 and *M. mucogenicum* (electron-accepting component – 0 mJ m^{-2}), had the ability to accept electrons (electron-accepting component between 0.6 mJ m^{-2} and 10.6 mJ m^{-2}). *Staphylococcus* sp. was one of the less hydrophilic bacteria and had the highest ability for accepting electrons. *A. calcoaceticus* was the less hydrophilic bacteria but had a very low electron donor capacity. *C. acidovorans* sp. 1 and *M. mucogenicum* were the more hydrophilic and were solely electron donors.

4.3.2 Characterization of colonisable materials

A range of materials were characterized in terms of surface properties (Table 4.2).

Table 4.2 Contact angle (in degrees), with water (θ_W), formamide (θ_F), α-bromonaphtalene (θ_B), surface tension parameters and free energy of interaction (ΔG_{sws}^{TOT}) of the support materials (s) when immersed in water (w). Values are means ± SDs

Support material	Contact angle (º)			Surface tension parameters (mJ m^{-2})			Hydrophobicity (mJ m^{-2})
	θ_W	θ_F	θ_B	γ_s^{LW}	γ_s^+	γ_s^-	ΔG_{sws}^{TOT}
SS 316	92.9±1.8	80.0±1.9	51.0±2.7	29.5	0.0	5.7	-55.1
SS 304	101±2.0	84.4±2.0	46.5±1.3	31.7	0.0	2.6	-71.5
Copper	98.6±3.5	77.7±2.2	45.4±2.0	32.1	0.0	1.5	-79.6
PVC	95.4±2.9	84.0±1.6	41.6±2.0	33.9	0.0	5.8	-55.9
PP	107±3.0	91.8±2.4	53.2±2.3	28.4	0.0	1.7	-76.6
PE	102±2.4	78.0±2.8	35.8±1.8	36.4	0.0	0.6	-90.3
Silicone	122±1.8	112±1.1	86.6±1.9	12.4	0.0	0.9	-85.6
Glass	73.5±3.1	68.9±3.1	50.8±1.6	29.6	0.0	20	-13.8

The water contact angle values of all the materials analyzed were higher than 65º and $\Delta G_{sws}^{TOT} < 0$, meaning that all the surfaces analyzed were hydrophobic with an electron donating character. In agreement with previous studies, (Flint *et al.*, 2000; Teixeira *et al.*, 2005) energy interaction values revealed that glass was the least hydrophobic of the test materials, while the most hydrophobic were PE and silicone. The remaining materials had water contact angle values that ranged between 92º and 107º. It was found that for SS 316 and SS 304 the water contact angle values (93º and 101º, respectively) were slightly higher than those reported in other studies (Flint *et al.*, 2000; Teixeira *et al.*, 2005), with a difference of approximately 10º for SS 316 and 15º for SS 304. Such differences could arguably be related to variations in surface finishing or the cleaning treatment. In accordance with the work performed by Sinde and Carballo (2000), it was found that SS 304 was more hydrophobic than SS 316, according to both the water contact angle and by the ΔG_{sws}^{TOT} value.

4.3.3 Prediction of adhesion

In order to predict the ability of the microorganisms to adhere to surfaces, the free energy of interaction between the isolated microorganisms and the materials, when immersed in water, was calculated (Table 4.3) according to the approach of van Oss *et al.* (1987, 1988, 1989). Based on this approach, *C. acidovorans* sp.1, *M. mucogenicum* and *Staphylococcus* sp. had no theoretical thermodynamic ability to adhere to the test materials, whilst adhesion was thermodynamically favorable at least to one material for the other bacteria. Comparing the thermodynamic ability for adhesion between test materials, it is noticeable that the adhesion is thermodynamically less favorable for glass due to its hydrophobic properties. Teixeira *et al.* (2005), also conclude that glass was a thermodynamically less favorable substrate for bacterial adhesion using a variety of dairy isolates in conjunction with materials commonly used in a dairy industry.

Adhesion to SS 316 and PVC, were thermodynamically favorable for six bacteria, SS 304 for 18 bacteria, copper, PP and PE for 21 bacteria, whilst silicone supported the theoretical adhesion of 22 bacteria. These data demonstrate that adhesion is dependent on the physicochemical properties of the bacterial surface and of the materials.

Table 4.3 Free energy of adhesion (ΔG_{bws}^{TOT}) between the isolated microorganisms (b) and the different support materials (s) when immersed in water (w)

Bacteria		ΔG_{bws}^{TOT} (mJ m^{-2})							
		SS 316	SS 304	Copper	PVC	PP	PE	Silicone	Glass
Acinetobacter calcoaceticus		-12.3	-17.5	-20.0	-12.2	-19.4	-23.1	-21.4	1.2
Burkholderia sp.		1.0	-3.8	-6.2	1.2	-5.6	-8.9	-7.9	13.5
Burkholderia cepacia		6.1	-0.1	-3.2	5.9	-2.1	-7.1	-2.7	22.0
CDC gr. IV C-2	sp. 1	8.0	1.3	-2.1	7.8	-0.8	-6.3	-1.2	25.1
	sp. 2	6.8	0.9	-2.0	6.6	-1.0	-5.6	-1.8	21.6
Comamonas acidovorans	sp. 1	30.2	22.0	18.0	29.9	19.4	12.9	18.5	50.8
	sp. 2	-14.3	-22.6	-26.7	-14.9	-24.9	-32.1	-24.1	6.4
	sp. 3	4.2	-0.7	-3.3	4.9	-3.0	-5.9	-7.2	17.9
	sp. 4	4.4	-1.5	-4.5	4.4	-3.5	-8.1	-4.6	19.6
Methylobacterium sp.		5.3	-0.8	-3.8	4.9	-2.5	-7.8	-1.8	20.5
Methylobacterium mesophilicum	sp. 1	-0.7	-7.9	-11.4	-1.5	-9.6	-16.2	-7.6	16.8
	sp. 2	1.6	-5.2	-8.6	1.2	-7.1	-13.0	-6.7	18.6
	sp. 3	4.3	-1.6	-4.4	3.9	-3.2	-8.2	-2.6	18.8
	sp. 4	0.2	-6.4	-9.7	0.0	-8.6	-13.8	-9.5	17.0
	sp. 5	5.0	-1.1	-4.1	4.6	-2.8	-8.0	-2.3	20.1
	sp. 6	0.4	-5.2	-7.9	0.2	-6.9	-11.4	-7.5	14.4
Moraxella lacunata		1.5	-2.5	-4.5	2.2	-4.5	-6.5	-8.8	12.6
Mycobacterium mucogenicum		17.2	9.3	5.3	17.4	6.4	0.7	3.5	37.9
Pseudomonas sp.		-2.7	-7.4	-9.9	-1.5	-10.1	-11.9	-17.0	11.0
Pseudomonas reactans		5.4	0.2	-2.3	5.5	-1.6	-5.4	-3.4	18.8
Sphingomonas capsulata	sp. 1	-12.1	-18.8	-22.1	-12.3	-20.9	-26.4	-21.2	5.0
	sp. 2	6.6	3.3	1.6	7.7	1.2	0.3	-4.7	16.3
Staphylococcus sp.		9.1	6.6	5.3	9.8	5.2	4.2	1.4	16.5
Stenotrophomonas maltophilia	sp. 1	-12.6	-19.1	-22.3	-12.9	-21.2	-26.4	-21.7	3.8
	sp. 2	3.5	-0.2	-2.1	3.9	-1.8	-4.0	-4.7	13.5

Comparing the predicted adhesion (Table 4.3) with the surface characteristics of the materials (Table 4.2), it can be seen that adhesion is favored as the surface hydrophobicity increases (linear correlation - $R^2 > 0.9$). Additionally, adhesion was favored when both surfaces are hydrophobic. This result is in agreement with other reports (Busscher *et al.*, 1990; Erner and Douglas, 1992; Flint *et al.*, 1997; Panagoda *et al.*, 1998; Chen and Strevett,

2001; Chavant *et al.*, 2002; Chen and Zhu, 2005). These investigations demonstrated a relationship between the surface properties and the extent of adhesion to solid materials. The current investigation showed that, based on surface tension parameters values, smaller γ_b^- values of both bacteria and surfaces lead to a thermodynamically favored adhesion (Tables 4.1 to 4.3). Considering the surface tension properties (Tables 4.1 and 4.2), both bacteria and support materials are electron donors. However, no apparent relationship between other surface tension parameters and thermodynamic adhesion was evident ($R^2 < 0.9$).

4.3.4 Adhesion to materials

Adhesion assays were performed with representative test bacteria and material surfaces, using a modified microtiter-plate assay methodology (Stepanović *et al.*, 2000). The bacteria used were *A. calcoaceticus*, *B. cepacia*, *Methylobacterium* sp., *M. mucogenicum*, *Sph. capsulata*, *Staphylococcus* sp., *S. maltophilia* and the control materials tested were SS 316, PVC and PE (Figure 4.1). Strain variation on adherence ability was assessed by using two distinct strains of *Sph. capsulata* (sp. 1 and sp. 2) and *S. maltophilia* (sp. 1 and sp. 2). Figure 4.1 shows that all tested bacteria adhered to the three distinct surfaces with different adhesion potentials.

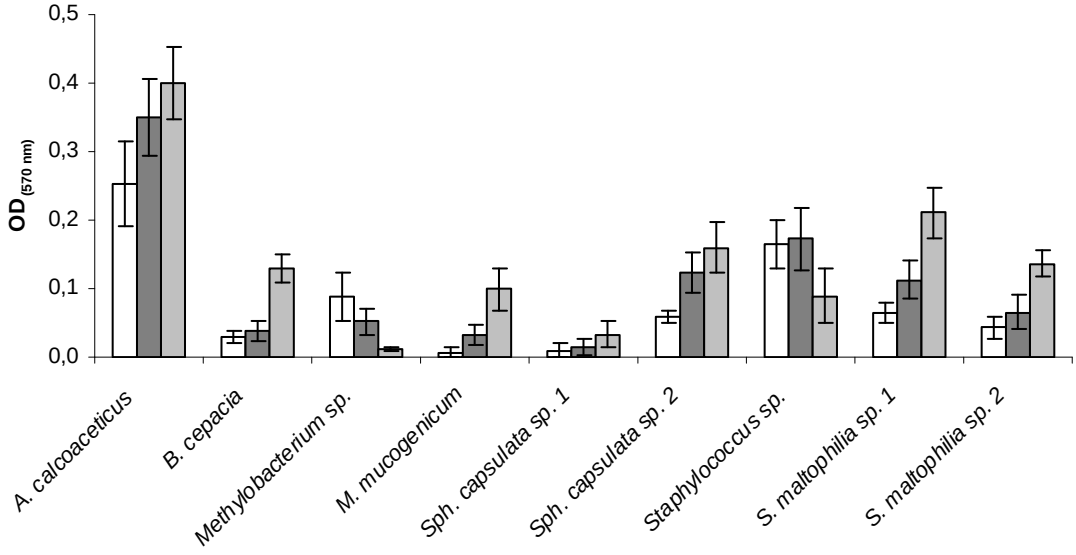

Figure 4.1 Values of $OD_{(570\ nm)}$ as a measure of adhesion of representative bacteria isolated from DW to SS 316 (□), PVC (■) and PE (□). The means ± SDs for at least three replicates are illustrated.

The tested bacteria adhered in a significant higher extent to PE ($P < 0.05$), with the exception of *Methylobacterium* sp. which adhered most strongly to SS 316 and *Staphylococcus* sp. (SS 316 and PVC). Furthermore, SS 316 was the material displaying the smaller number of adhered cells ($P < 0.05$), except for *Methylobacterium* sp. and *Staphylococcus* sp. Also, the adherence ability of *Sph. capsulata* sp. 1 to SS 316 and PVC, *M. mucogenicum* to SS 316 and *Methylobacterium* sp. to PE was almost negligible. *A. calcoaceticus* had the highest ability to adhere to the three control materials, while *Sph. capsulata* sp. 1 was the bacterium with the lowest adherence ability. Comparing adhesion ability within the same species, a significant difference ($P < 0.05$) was detected for *Sph. capsulata* strains, whilst for *S. maltophilia* strains the differences were smaller ($P > 0.05$), although *S. maltophilia* sp. 1 possessed higher ability to adhere to the three control materials.

A rank of adherence was produced according to Stepanović *et al.* (2000), classifying test bacteria as non-adherent, weakly adherent, moderately adherent and strongly adherent bacteria (Table 4.4).

Table 4.4 Adhesion ability of representative bacteria isolated from DW to SS 316, PVC and PE[a]

Bacteria		Adhesion surfaces		
		SS 316	PVC	PE
Acinetobacter calcoaceticus		++	+++	+++
Burkholderia cepacia		0	0	+
Methylobacterium sp.		+	0	0
Mycobacterium mucogenicum		0	0	+
Sphingomonas capsulata	sp. 1	0	0	0
	sp. 2	0	+	+
Staphylococcus sp.		+	++	+
Stenotrophomonas maltophilia	sp. 1	0	+	++
	sp. 2	0	0	+

[a]According to the adherence classification proposed by Stepanović *et al.* (2000): (0) non-adherent; (+) weakly adherent; (++) moderately adherent; (+++) strongly adherent

A. calcoaceticus was the only strongly adherent microorganism to PVC and PE. Moderate adherence was detected for *A. calcoaceticus* to SS 316, *Staphylococcus* sp. to PVC and *S. maltophilia* sp. 1 to PE. Weak adherence was observed for *Methylobacterium*

sp. and *Staphylococcus* sp. to SS 316, *S. maltophilia* sp. 1 and *Sph. capsulata* sp. 2 to PVC, *B. cepacia, M. mucogenicum, Sph. capsulata* sp. 2, *Staphylococcus* sp. and *S. maltophilia* sp. 2 to PE. The remaining bacterial/material situations analyzed demonstrated that bacteria were non-adherent to the three materials. The use of distinct *S. maltophilia* and *Sph. capsulata* strains showed the existence of varying ability of adherence for the distinct strains, suggesting that individual strains are not reliable predictive paradigms (Fux *et al.*, 2005).

4.3.5 Comparison between thermodynamic prediction and adhesion assay

Comparison between the theoretical thermodynamic prediction of adhesion (Table 4.3) and laboratory adhesion assays (Figure 4.1) shown that adhesion is underestimated when based on thermodynamic approaches. In fact, the results were only in agreement for *A. calcoaceticus, B. cepacia* and *S. maltophilia* sp. 2 (Table 4.4). No agreement between thermodynamic approaches and the adhesion assays was obtained for *Sph. capsulata* sp. 1 and *Staphylococcus* sp. A similar trend between thermodynamic prevision and adhesion results was found for *M. mucogenicum* when assayed with SS 316 and PVC, *S. maltophilia* sp. 1 when assayed with PVC and PE, *Methylobacterium* sp. when assayed with PVC and *Sph. capsulata* sp. 2 when assayed with SS 316.

The apparent agreement between thermodynamic and adhesion results detected for *A. calcoaceticus, B. cepacia* and *S. maltophilia* sp. 2, suggests the existence of a strong influence of surface physicochemical properties on bacterial adhesion for the referred bacteria. Slight correlation was found between the bacterial surface properties and the adhesion results ($R^2 < 0.85$). For all the bacteria, the extent of adhesion correlated with all surface properties of the materials ($R^2 \geq 0.85$), verifying that adhesion increases with the decrease of γ_s^- and with the increase of γ_s^{LW} and hydrophobicity (ΔG_{sws}^{TOT}) of the materials, with the exception of *Methylobacterium* sp. and *Staphylococcus* sp. Several previous studies have reported the lack of a correlation between hydrophobicity of the bacteria and bacterial attachment; the attachment process was strongly influenced by the presence of extracellular molecules (Li and Logan, 2004; Chae *et al.*, 2006). Sardin *et al.* (2004), however, reported a correlation between bacterial adherence with the non-polar surface tension component of the materials and with bacterial hydrophobicity. In the current study, the lack of agreement

between thermodynamic approaches and adhesion assays reinforces that biological mechanisms, such as the expression of adhesins that mediate specific interactions with substrata at a nanometer scale (during the irreversible phase of microbial adhesion) in addition to the physicochemical ones, mediate the entire microbial adhesion process (Flint *et al.*, 1997; Doyle, 2000; Sinde and Carballo, 2000).

In conclusion, whilst the prediction of adhesion potential on the basis of physicochemical properties gives useful information about the possible real-life microbial behaviour, adhesion results suggest that mechanisms other than cellular physicochemical surface properties may play a determinant role on bacterial adherence ability. These will include microbial *flagella*, *pili* or *fimbrae*, *prothecae* and production of extracellular polymeric substances (Flint *et al.*, 1997; Doyle, 2000; Sinde and Carballo 2000; Donlan, 2002; Simões, 2005). Prediction based on surface physicochemical properties and thermodynamic approaches therefore did not provide conclusive results. Furthermore, because multispecies interactions prevail in the environment, strongly adherent bacteria may play a determinant role in the primary colonization of surfaces, seeding biofilms which will then develop by cellular proliferation and immigration.

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CHAPTER 5 ADHESION AND BIOFILM FORMATION BY DRINKING WATER BACTERIA

This study was performed in order to characterize the relationship between adhesion and biofilm formation abilities of DW-isolated bacteria (*Acinetobacter calcoaceticus*, *Burkholderia cepacia*, *Methylobacterium* sp., *Mycobacterium mucogenicum*, *Sphingomonas capsulata* and *Staphylococcus* sp.). Adhesion was assessed by two distinct methods: thermodynamic prediction of adhesion potential by quantifying hydrophobicity and the free energy of adhesion; and by microtiter plate assays. Biofilms were developed in microtiter plates during 24, 48 and 72 h. Polystyrene (PS) was used as adhesion substratum. The tested bacteria had negative surface charge and were hydrophilic. PS had negative surface charge and was hydrophobic. The free energy of adhesion between the bacteria and PS was > 0 mJ m^{-2} (thermodynamic unfavorable adhesion). The thermodynamic approach was inappropriate for modelling adhesion of the tested DW bacteria, underestimating adhesion to PS. Only three (*B. cepacia*, *Sph. capsulata* and *Staphylococcus* sp.) of the six bacteria were non-adherent to PS. *A. calcoaceticus*, *Methylobacterium* sp. and *M. mucogenicum* were weakly adherent. This adhesion ability was correlated with the biofilm formation ability when comparing with the results of 24 h aged biofilms. *Methylobacterium* sp. and *M. mucogenicum* formed large biofilm amounts, regardless the biofilm age. Given time, all the bacteria formed biofilms; even those non-adherents produced large amounts of matured (72 h aged) biofilms. The overall results indicate that initial adhesion did not predict the ability of the tested DW-isolated bacteria to form a mature biofilm, suggesting that other events such as phenotypic and genetic switch during biofilm development and the production of EPS, may play a significant role on biofilm formation and differentiation. This understanding of the relationship between adhesion and biofilm formation is important for the development of control strategies efficient in the early stages of biofilm development.

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5.1 Introduction

Many problems in DWDS are related with the presence of microorganisms, including biofilm growth, nitrification, microbially mediated corrosion, and the occurrence and persistence of pathogens (Regan *et al.*, 2003; Camper, 2004; Emtiazi *et al.*, 2004; Bauman *et al.*, 2009). DWDS are known to harbour biofilms, even though these environments are oligotrophic and often contain a disinfectant. By adopting this sessile mode of life, biofilm-embedded microorganisms enjoy a number of advantages over their planktonic counterparts, namely the increased resistance to antimicrobials (Gilbert *et al.*, 2002). Microbial adhesion will initiate biofilm formation, exacerbating contamination of DW, reducing the aesthetic quality of potable water, increasing the corrosion rate of pipes and reducing microbiological safety through increased survival of pathogens (Percival and Walker, 1999; Niquette *et al.*, 2000). The development of a biofilm is believed to occur in a sequential process that includes transport of microorganisms to surfaces, initial reversible/irreversible adhesion, cell-cell communication, formation of microcolonies, EPS production and biofilm maturation (Doyle, 2000; Sauer and Camper, 2001; Bryers and Ratner, 2004; Dobretsov *et al.*, 2009). Accordingly, the adhesion of bacteria to the surface is one of the prime steps in biofilm formation.

Several theoretical approaches have been applied to describe bacteria-surface adhesion, such as the classical DLVO theory (Rutter and Vincent, 1984; van Loosdrecht *et al.*, 1988), the extended DLVO (XDLVO) theory (van Oss, 1989; Meinders *et al.*, 1995), and the thermodynamic approach (surface Gibbs energy) (Absolom *et al.*, 1983; Busscher *et al.*, 1984). When a microorganism and a surface in aqueous solution enter in direct contact the water film present between the interacting entities has to be removed. This is in accordance with the thermodynamic theory of adhesion and is expressed by the Dupré equation which states that the Gibbs free energy of interaction can be calculated assuming that the interfaces between bacteria/liquid medium and solid/liquid medium are replaced by a bacteria/solid interface (Absolom *et al.*, 1983). The interaction between a microbial cell and a solid substratum is only possible from a thermodynamic point of view if it leads to a decrease in the surface Gibbs free energy (Absolom *et al.*, 1983; Busscher *et al.*, 1984). Those approaches consider bacteria as colloids. However, important biological factors have been largely ignored in those models. Walker *et al.* (2004, 2005) have found that the

heterogeneity of active sites from cell surface macromolecules, such as proteins and lipopolysaccharide-associated functional groups, controls the adhesion process.

Bacterial adhesion is a complex process that is affected by many factors, including the physicochemical characteristics of bacteria (hydrophobicity, surface charge), the material surfaces properties (chemical composition, surface charge, hydrophobicity, roughness and texture) and by the environmental factors (temperature, pH, time of exposure, bacterial concentration, chemical treatment or the presence of antimicrobials and fluid flow conditions). The biological properties of bacteria, such as the presence of fimbriae and flagella, and the production of EPS also influence the attachment to surface (An and Friedman, 1998). Recently, adhesion has been described as a two-phase process including an initial, instantaneous, and reversible physicochemical phase and a time-dependent and irreversible molecular and cellular phase (Pavithra and Doble, 2008). In the first phase, planktonic bacteria move or are moved to a surface through and by the effects of physical forces, such as Brownian motion, van der Waals attraction forces, gravitational forces, the effect of surface electrostatic charge, and hydrophobic interactions. These physical interactions are further classified as long-range (non-specific, distances > 150 nm) and short-range interactions (distances < 3 nm). Bacteria are first transported to the surface by the long-range interactions and at closer proximity the short-range interactions become more important. In the second phase, molecular reactions between bacterial surface structures and substratum surfaces become predominant. This implies a firmer adhesion of bacteria to a surface by the bridging function of bacterial surface polymeric structures.

The understanding of the overall biofilm formation process depends on the deep understanding of the main aspects regulating biofilm development, such as the initial adhesion. However, there is a lack of information regarding the behaviour of cells in the earlier stages of biofilm formation, and its relationship with the biofilm development process. This study was performed in order to characterize the adhesion and biofilm formation abilities of DW-isolated bacteria to PS and to assess the possible relationships between adhesion and biofilm results.

5.2 Material and methods

5.2.1 Bacteria isolation and identification

The microorganisms used throughout this work were isolated from a model laboratory DWDS, as described previously in chapter 3 and by Simões *et al.* (2006). Identification tests, by determination of 16S rDNA gene sequence, were performed for putative bacteria according to the method described in chapter 4 and by Simões *et al.* (2007a).

5.2.2 Planktonic bacterial growth

Assays were performed with six representative (above 80% of the total bacterial genera isolated and identified) DW bacteria: *Acinetobacter calcoaceticus*, *Burkholderia cepacia, Methylobacterium sp., Mycobacterium mucogenicum, Sphingomonas capsulata* and *Staphylococcus sp.*

Bacterial cells were grown overnight in batch culture using 100 ml of R2A (Merck, Portugal) broth, at room temperature (23 ºC ± 2), under agitation (150 rpm). Cells were harvested by centrifugation (20 min at 13000 g), washed three times in PBS (0.1 M, pH 7.2) and resuspended in a certain volume of sterile tap water (pH 6.7 ± 0.2) or R2A broth (biofilm studies) necessary to achieve the bacterial concentration required for each assay.

5.2.3 Substratum

The material assayed was PS. In order to prepare PS for further analysis, it was immersed in a solution of commercial detergent (Sonasol Pril, Henkel Ibérica S. A.) and ultrapure water for 30 min. In order to remove any remaining detergent, the material was rinsed in ultrapure water and subsequently immersed in ethanol at 96% (v v^{-1}) for 10 s. After being rinsed three times with ultrapure water, it was dried at 65 ºC for 3 h before being used for contact angle measurements, zeta potential assessment and adhesion assays.

5.2.4 Zeta potential

Zeta potential experiments were performed with the cells ressuspended in sterile tap water at a final concentration of 10^9 cells ml^{-1}. The zeta potential of PS was also assessed. The experiments were determined using a Malvern Zetasizer instrument (Zetasizer Nano ZS ZEN3600, Malvern). Before measuring the electrostatic values, the zeta potential cell (DTS1060, Malvern) was rinsed three times with each suspension using a disposable syringe. All experiments were carried out at room temperature. The zeta potential was derived from the electrophoretic mobility using the Smoluchowski approximation (Hunter, 1981). The experiments were performed in triplicate and repeated three times.

5.2.5 Surface contact angles

Bacterial lawns for contact angle measurements were prepared as described by Busscher *et al.* (1984). The surface tension of the bacterial surfaces and of the adhesion surface were then determined using the sessile drop contact angle method. The measurements were carried out at room temperature using three different liquids: water, formamide and α-bromonaphtalene (Sigma, Portugal). 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 25 determinations for each liquid and for each microorganism and PS) were performed at three independent experiments for each condition tested. The reference liquids surface tension components were obtained from literature (Janczuk *et al.*, 1993).

5.2.6 Surface hydrophobicity and free energy of adhesion

Hydrophobicity was assessed after contact angle measurements and using the approach of van Oss *et al.* (1987, 1988, 1989). In this approach, the degree of hydrophobicity of a given material (1) is expressed as the free energy of interaction between two entities of that material when immersed in water (w) - ΔG_{1w1}. If the interaction between the two entities is stronger than the interaction of each entity with water $\Delta G_{1w1} < 0$

mJ m^{-2} the material is considered hydrophobic. Conversely, if $\Delta G_{1w1} > 0$ the material is hydrophilic. ΔG_{1w1} can be calculated through the surface tension components of the interacting entities, according to:

$$\Delta G_{1w1} = -2\left(\sqrt{\gamma_1^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_1^+\gamma_w^-} + \sqrt{\gamma_1^-\gamma_w^+} - \sqrt{\gamma_1^+\gamma_1^-} - \sqrt{\gamma_w^+\gamma_w^-}\right) \quad (1)$$

where γ^{LW} accounts for the Lifshitz-van der Waals component of the surface free energy and γ^+ and γ^- are the electron acceptor and electron donor parameters, respectively, of the Lewis acid-base component (γ^{AB}), with $\gamma^{AB} = 2\times\sqrt{\gamma^+\gamma^-}$.

The surface tension components of a surface (s) (bacteria or substratum) are obtained by measuring the contact angles of three pure liquids (l) (one apolar - α-bromonaphtalene and two polar – water and formamide), with well known surface tension components, followed by the simultaneous resolution of three equations of the form:

$$(1+\cos\theta)\gamma_1^{TOT} = 2\left(\sqrt{\gamma_s^{LW}\gamma_1^{LW}} + \sqrt{\gamma_s^+\gamma_1^-} + \sqrt{\gamma_s^-\gamma_1^+}\right) \quad (2)$$

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

The free energy of adhesion was calculated through the surface tension components of the entities involved in the adhesion process by the thermodynamic theory expressed by Dupré equation (3). When studying the interaction between one bacteria (b) and a substratum (s) that are immersed or dissolved in water (w), the total interaction energy, ΔG_{bws}^{TOT}, can be expressed by the interfacial tensions components as:

$$\Delta G_{bws}^{TOT} = \gamma_{bs} - \gamma_{bw} - \gamma_{sw} \quad (3)$$

For instance, the interfacial tension for one diphasic system of interaction (bacteria/substratum - γ_{bs}) can be defined by the thermodynamic theory according to the following equations:

$$\gamma_{bs} = \gamma_{bs}^{LW} + \gamma_{bs}^{AB} \quad (4)$$

$$\gamma_{bs}^{LW} = \gamma_b^{LW} + \gamma_s^{LW} - 2\times\sqrt{\gamma_b^{LW}\times\gamma_s^{LW}} \quad (5)$$

$$\gamma_{bs}^{AB} = 2\times\left(\sqrt{\gamma_b^+\times\gamma_b^-} + \sqrt{\gamma_s^+\times\gamma_s^-} - \sqrt{\gamma_b^+\times\gamma_s^-} - \sqrt{\gamma_b^-\times\gamma_s^+}\right) \quad (6)$$

The other interfacial tension components, γ_{bw} (bacteria/water) and γ_{sw} (substratum/water), were calculated in the same way. The value of the free energy of adhesion was obtained by the application of Equations 3 to 6, which allowed the assessment

of thermodynamic adhesion. Thermodynamically, if $\Delta G_{bws}^{TOT} < 0$ mJ m^{-2} the adhesion of one bacteria to substratum is favourable. On the contrary, adhesion is not expected to occur if $\Delta G_{bws}^{TOT} > 0$ mJ m^{-2}.

5.2.7 Adhesion

Coupons of PS with 8 mm × 8 mm, prepared as indicated previously, were inserted in the bottom of 24-wells (15 mm diameter each well) microtiter plates (polystyrene, Orange Scientific, USA) and 2 ml of each cell suspension (10^9 cells ml^{-1} in sterile tap water), was added to each well. Adhesion to each material was allowed to occur for 2 h at room temperature, in an orbital shaker at 150 rpm, according to the methods of Simões *et al.* (2007a). Negative controls were obtained by placing PS in sterile tap water without bacterial cells. At the end of the assay each well was washed twice with sterile distilled water, by pipetting carefully only the liquid above the coupon to remove reversibly adherent bacteria. After the last wash, the coupons were used for biomass quantification by CV staining. All the experiments were performed in triplicate with three repeats.

5.2.8 Biofilm formation

Biofilms were developed according to the modified microtiter plate test proposed by Stepanović *et al.* (2000). Briefly, for each bacterium at least sixteen wells of a sterile 96-well flat tissue culture plates (polystyrene, Orange Scientific, USA) were filled under aseptic conditions with 200 μl of cell suspension (10^8 cells ml^{-1} in R2A broth). To promote biofilm formation, the plates were incubated aerobically on a shaker at 150 rpm, at room temperature, for 24, 48 and 72 h. Each 24 h the growth medium was carefully discarded and replaced by fresh one. After each biofilm formation period, the content of each well was removed and the wells were washed three times with 250 μl of sterile distilled water to remove reversibly adherent bacteria. The plates were air dried for 30 min, and the remaining attached bacteria were analysed in terms of biomass adhered on the surfaces of the microtiter plates. Negative controls were obtained by incubating the wells only with R2A broth without adding any bacterial cells. All the experiments were repeated three times.

5.2.9 Biomass quantification by CV

The coupons with adhered bacteria in the 24-wells plates were removed from each well and immersed in a new microtiter plate containing 1 ml of methanol 98% (v v^{-1}) in each well for biomass quantification by CV (Gram-colour-staining set for microscopy, Merck) (Simões *et al.*, 2007a). Methanol was withdrawn after 15 min of contact and the coupons were allowed to dry at room temperature. Aliquots (600 µl) of CV were then added to each well and incubated for 5 min. After gently washing in water the coupons were left to dry, before being immersed in 1 ml of acetic acid 33% (v v^{-1}) to release and dissolve the stain.

The bacterial biofilms in the 96-wells plates were fixed with 250 µl of 98% methanol (Vaz Pereira, Portugal) per well for 15 min. Afterwards, the plates were emptied and left to dry. Then, the fixed bacteria were stained for 5 min with 200 µl of CV per well. Excess stain was rinsed off by placing the plate under running tap water (Stepanović *et al.*, 2000). After the plates were air dried, the dye bound to the adherent cells was resolubilized with 200 µl of 33% (v v^{-1}) glacial acetic acid (Merck, Portugal) per well.

The OD of the obtained solutions were measured at 570 nm using a microtiter plate reader (BIO-TEK, Model Synergy HT) and adhesion and biofilm mass were presented as OD_{570nm} values.

5.2.10 Adherent/biofilm bacteria classification

Bacteria were classified using the scheme of Stepanović *et al.* (2000) as follow: non-adherent/non-biofilm producer (0): OD ≤ ODc; weakly adherent/weak biofilm producer (+): ODc < OD ≤ 2 × ODc; moderately adherent/moderate biofilm producer (++): 2 × ODc < OD ≤ 4 × ODc; strongly adherent/strong biofilm producer (+++): 4 × ODc < OD. This classification was based upon the cut-off of the OD (ODc) value defined as three standard deviation values above the mean OD of the negative control.

5.2.11 Statistical analysis

The data were analysed using the statistical program SPSS version 14.0. Because low samples numbers contributed to uneven variation, the adhesion results were analyzed by

the nonparametric Wilcoxon test. Statistical calculations were based on a confidence level of ≥ 95% ($P < 0.05$ was considered statistically significant).

5.3 Results

5.3.1 Surface physicochemical properties and free energy of adhesion

Bacterial adhesion can be influenced by the surface physicochemical properties of both bacteria and substratum. Consequently, the DW-isolated bacteria and the PS surface were characterized in terms of surface properties - hydrophobicity and surface charge (zeta potential). All the tested isolates had negative zeta potential. The bacteria with the highest zeta potential was *A. calcoaceticus* (-6.7 ± 0.4 mV) and *M. mucogenicum* (-31 ± 3 mV) had the lowest zeta potential (Table 5.1). PS surface had a zeta potential of -32 ± 2 mV (Table 5.1).

Table 5.1 Zeta potential (mV) values of DW-isolated bacteria and PS. Values are means ± SDs of three independent experiments

	Zeta Potential (mV)
Bacteria	
Acinetobacter cacoaceticus	-6.7 ± 0.4
Burkholderia cepacia	-7.7 ± 0.3
Methylobacterium sp.	-9.0 ± 0.5
Mycobacterium mucogenicum	-31 ± 3
Sphingomonas capsulata	-27 ± 0.6
Staphylococcus sp.	-10 ± 0.3
Substratum	
PS	-32 ± 2

The surface hydrophobicity was determined as a quantitative result using the approach proposed by van Oss (1995, 1997), which allows the assessment of the absolute degree of hydrophobicity of any surface in comparison with their interaction with water. Based on this approach the surfaces of the tested bacteria are hydrophilic ($\Delta G_{bwb}^{TOT} > 0$ mJ m^{-2}) (Table 5.2). Conversely, the PS surface is hydrophobic ($\Delta G_{sws}^{TOT} = -44$ mJ m^{-2}) (Table

5.2). Bacteria had similar hydrophobicity values ($P > 0.05$), with the exception of *Sph. capsulata*. According to the surface tension parameters (Table 5.2), the Lifshitz-van der Waals (γ^{LW}) component of the bacteria had similar values and all the bacteria were predominantly electron donors (γ^-). Moreover, all the bacteria had the ability to accept electrons (γ^+). On the other hand, PS had only an electron donating character (γ^+ = 0 mJ m^{-2}).

Table 5.2 Contact angles (in degrees) with water (θ_W), formamide (θ_F), α-bromonaphtalene (θ_B), surface tension parameters, free energy of interaction (ΔG^{TOT}_{bwb} or ΔG^{TOT}_{sws}) of the bacteria (b) and PS (s) when immersed in water (w); free energy of adhesion (ΔG^{TOT}_{bws}) between the bacteria (b) and PS (s) when immersed in water (w). Values are means ± SDs of three independent experiments

	Contact angle (º)			**Surface tension parameters (mJ m^{-2})**			**Hydrophobicity (mJ m^{-2})**	**Free energy of adhesion (mJ m^{-2})**
	θ_W	θ_F	θ_B	γ^{LW}	γ^+	γ^-	ΔG^{TOT}_{bwb} **or** ΔG^{TOT}_{sws}	ΔG^{TOT}_{bws}
Bacteria								
Acinetobacter calcoaceticus	28±1	31±1	43±0.8	33	1.3	51	30	2.3
Burkholderia cepacia	38±2	43±2	47±1	32	0.5	49	32	0.3
Methylobacterium sp.	20±1	20±2	42±2	34	2.1	51	28	4.1
Mycobacterium mucogenicum	27±1	25±1	58±8	26	4.4	46	20	5.3
Sphingomonas capsulata	31±5	53±2	73±4	19	1.2	69	51	19
Staphylococcus sp.	28±0.9	27±1	51±2	30	2.8	47	23	3.0
Substratum								
PS	83±3	71±2	28±1	39	0.0	9.9	-44	-

ΔG^{TOT}_{bwb} or ΔG^{TOT}_{sws} < 0 mJ m^{-2} – hydrophobic surface; ΔG^{TOT}_{bwb} or ΔG^{TOT}_{sws} > 0 mJ m^{-2} – hydrophilic surface.
ΔG^{TOT}_{bws} < 0 mJ m^{-2} – thermodynamic favourable adhesion; ΔG^{TOT}_{bws} > 0 mJ m^{-2} – thermodynamic unfavorable adhesion.

In order to predict the ability of the microorganisms to adhere to PS surfaces, the free energy of interaction between the bacteria and the surface, when immersed in water, was calculated according to the thermodynamic approach. Based on this approach, all the bacteria had no theoretical thermodynamic ability to adhere to PS (ΔG^{TOT}_{bws} > 0 mJ m^{-2}). *B.*

cepacia had the smallest ΔG_{bws}^{TOT} and *Sph. capsulata* had the highest ΔG_{bws}^{TOT} (less prone to adhere to PS).

5.3.2 Adhesion

Adhesion assays were performed with the DW-isolated bacteria and PS surfaces, using a modified microtiter-plate assay methodology (Stepanović *et al.*, 2000) and CV staining for biomass assessment of the adhered bacteria. The tested bacteria adhered to PS surfaces (Figure 5.1) with different potentials ($P < 0.05$). *A. calcoaceticus* and *Sph. capsulata* had the highest and lowest adhesion ability, respectively. *Methylobacterium* sp. and *M. mucogenicum* adhered to similar extents ($P > 0.05$). The degree of bacterial adhesion was found to follow the sequence *A. calcoaceticus* > *Methylobacterium* sp. > *M. mucogenicum* > *Staphylococcus* sp. > *B. cepacia* > *Sph. capsulata*. However, only *A. calcoaceticus, Methylobacterium* sp. and *M. mucogenicum* were weakly adherent to PS. The remaining bacteria were classified as non-adherent (Table 5.3).

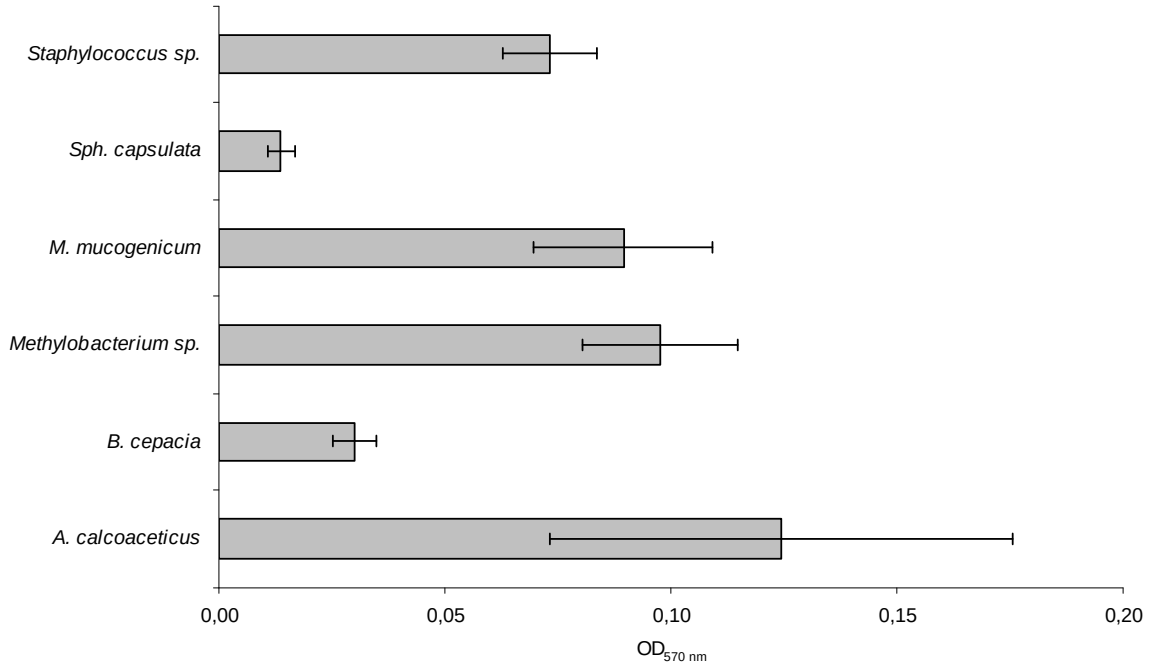

Figure 5.1 Values of $OD_{570\ nm}$ as a measure of bacteria adhesion to PS during 2 h. The means ± SDs for three independent experiments are illustrated.

Table 5.3 Adhesion and biofilm formation ability of DW-isolated bacteria to PS according to the classification proposed by Stepanović *et al.* (2000) and used by Simões *et al.* (2007b)

Bacteria	Adhesion	Biofilm		
		24 h	48 h	72 h
Acinetobacter calcoaceticus	+	+	0	+++
Burkholderia cepacia	0	0	+	+
Methylobacterium sp.	+	+++	+++	+++
Mycobacterium mucogenicum	+	+++	+++	+++
Sphingomonas capsulata	0	0	0	++
Staphylococcus sp.	0	0	0	++

(0) non-adherent/non-biofilm producer; (+) weakly adherent/weak biofilm producer; (++) moderately adherent/moderate biofilm producer; (+++) strongly adherent/strong biofilm producer

5.3.3 Biofilm formation

In order to assess the biofilm formation ability of the several DW-isolated bacteria, a standard 96-wells microtiter plates with CV staining was used to characterize biofilms (Figure 5.2). The tested bacteria formed biofilms, with *Methylobacterium* sp. producing the highest biomass amount for all the sampling times. *M. mucogenicum* was the second stronger biofilm producer. A directly proportional time - biomass formation was found for the various bacteria ($P < 0.05$), except for *B. cepacia* ($P > 0.05$). Only for sampling times higher than 48 h, *Sph. capsulata* formed biofilms. The degree of biofilm formation was found to follow the sequence – 24 h biofilms: *Methylobacterium* sp. > *M. mucogenicum* > *A. calcoaceticus* > *Staphylococcus* sp. > *B. cepacia* > *Sph. capsulata*; 48 h biofilms: *Methylobacterium* sp. > *M. mucogenicum* > *B. cepacia* > *Staphylococcus* sp. > *A. calcoaceticus* > *Sph. capsulata*; 72 h biofilms: *Methylobacterium* sp. > *M. mucogenicum* > *A. calcoaceticus* > *Staphylococcus* sp. > *Sph. capsulata* > *B. cepacia*.

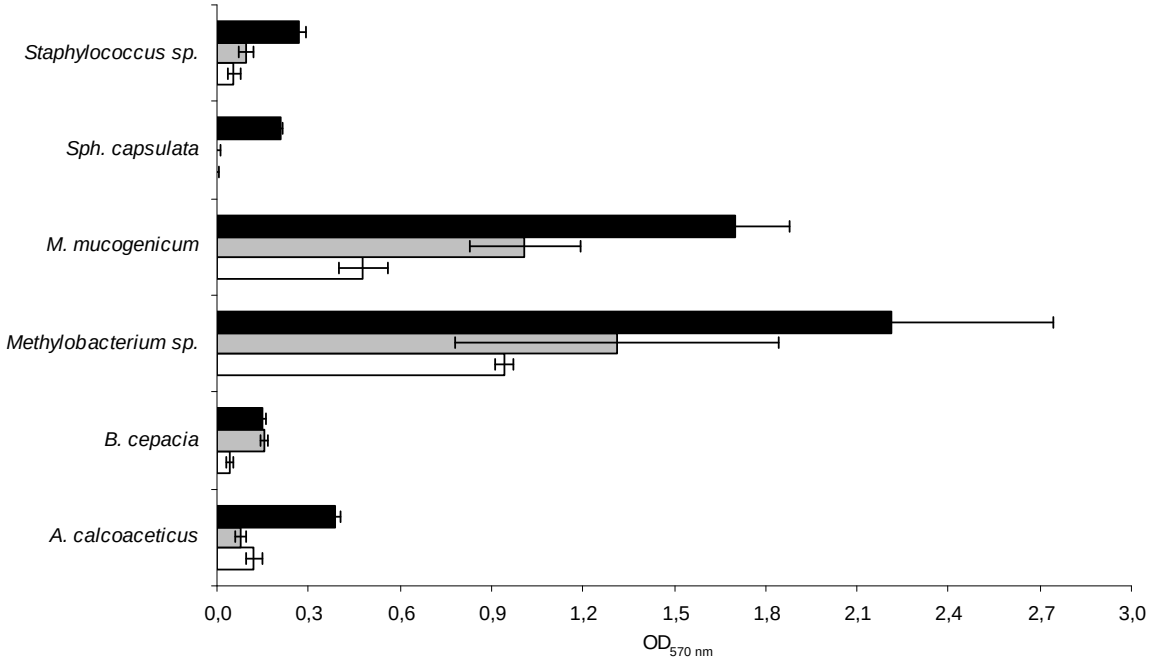

Figure 5.2 Values of $OD_{570\ nm}$ as a measure of mass of 24 h (□), 48 h (▩) and 72 h (■) aged biofilms. The means ± SDs for three independent experiments are illustrated.

According to the rank of biofilm formation (Table 5.3), *Methylobacterium* sp. and *M. mucogenicum* showed a strong biofilm producing ability for the several sampling times. *Sph. capsulata* and *Staphylococcus* sp. only presented biofilm formation ability (moderate) for the 72 h sampling time. *B. cepacia* formed weak biofilms after 48 h, while *A. calcoaceticus* showed variability in the biofilm formation ability by forming weak biofilms at 24 h, being classified as non-biofilm producer at 48 h, and as a strong biofilm producer at the 72 h sampling time.

5.4 Discussion

The dynamics of the microbial growth and biofilm formation in DW networks is very complex, as a large number of interacting processes are involved (Simões *et al.*, 2007b, 2008b; Liu *et al.*, 2009). Biofilms are suspected to be the primary source of microorganisms in DWDS that are fed with treated water and have no pipeline breaches, and are of particular concern in older DWDS (LeChevalier *et al.*, 1987). Bacterial adhesion to surfaces, the first step in the formation of a biofilm, has been studied extensively over the past decades in many diverse areas. However, to our knowledge this is the first study reporting the

relationship between adhesion and biofilm formation by autochthonous DW bacteria. Microorganisms isolated from any given niche, whether medical, environmental, water, or industrial, will have different mechanisms of adhesion and retention, not only because the substrata, nutrients, ionic strength, pH values, and temperatures differ, but also because their phenotype and genotype (expression of structural components and adhesive surface proteins) have adapted differently over time through selective pressures (Thomas *et al.*, 2002). Bakker *et al.* (2004) also reported that bacterial strains isolated from different niches can exhibit different patterns of adhesion to substrata. The bacteria used in this study are recognized as problematic opportunistic bacteria with the potential to cause public health problems (Bifulco *et al.*, 1989; Rusin *et al.*, 1997; Szewzyk *et al.*, 2000; Zanetti *et al.*, 2000; Conway *et al.*, 2002; Pavlov *et al.*, 2004; Stelma *et al.*, 2004). Similarly to other studies, PS was used as a model surface for adhesion and biofilm formation under laboratorial conditions (Simões *et al.*, 2007b; Pompilio *et al.*, 2008; Johansen *et al.*, 2009). The PS microtiter plates are commonly used as the standard bioreactor system for adhesion and biofilm formation of bacteria isolated from many different environments, providing reliable comparative data (Djordjevic *et al.*, 2002; Andersson *et al.*, 2008; Cotter *et al.*, 2009). PS has physicochemical surface properties (hydrophobicity) similar to those of other materials used in water distribution systems such as stainless steel and polyvinylchloride (Simões *et al.*, 2007a). Understanding the relationship between adhesion and biofilm formation is crucial to understand the role microorganisms may play in the system and to develop reliable preventive and control strategies efficient in the early stages of biofilm development.

The influence of the surface free energies of the substratum and the bacterium can be modelled using a thermodynamic approach (Bos *et al.*, 1999). The XDLVO theory accounts for Lifshitz–van der Waals, electrostatic and short range acid-base interaction energies between the surface and the bacterium as a function of their separation distance (van Oss *et al.*, 1986). This mechanistic knowledge of bacterial adhesion obtained from the XDLVO theory provides guidelines for the development of surface coatings exhibiting propensity for minimal bacterial adhesion (Genzer and Efimenko, 2006; Webster *et al.*, 2007; Bennett *et al.*, 2010). However, the initial microbial adhesion, as governed by physicochemical interaction forces, is only one of the steps in the development of a mature biofilm. After adsorption of conditioning film components and adhesion of initial colonizers, many subsequent biological, ecological and environmental events determine the ultimate

microbial composition and structure of a mature biofilm (Bryers and Ratner, 2004; Simões *et al.*, 2009).

Bacterial characteristics known to influence adhesion are hydrophobicity, surface charge, motility, and release of extracellular substances, such as polysaccharides, proteins and metabolite molecules (Dufrêne *et al.*, 1996; Kogure *et al.*, 1998; Azeredo *et al.*, 1999; Bos *et al.*, 1999; van Hoogmoed *et al.*, 2000). Relevant properties of the substratum surface are hydrophobicity, charge, and texture (Holland *et al.*, 1998; Bos *et al.*, 1999; Gottenbos *et al.*, 1999; Akesso *et al.*, 2009). Based on the surface properties studied all the bacteria had negative zeta potential and are hydrophilic. According to Rijnaarts *et al.* (1999), at physiological pH (pH 7) bacterial cells generally have a net negative charge on their cell wall. In this study, the bacteria had similar hydrophobicity (exception – *Sph. capsulata*) and zeta potential (exceptions – *M. mucogenicum* and *Sph. capsulata*) values. It is not surprising that the surface properties of *M. mucogenicum* were considerably different from the other bacteria due to the presence of a waxy cell wall. PS had also negative zeta potential, but had a hydrophobic character. Furthermore, it was observed that all bacteria were predominantly electron donors, with low electron acceptor parameters. This polar character can be due to the presence of residual water of hydration or polar groups (van Oss, 1994).

A comparison between the theoretical thermodynamic adhesion evaluation and the adhesion assays shows that adhesion was underestimated when based on thermodynamic approaches. In fact, no agreement between thermodynamic approaches and the adhesion assays were obtained for the tested bacteria. Even if for all the bacteria $\Delta G_{bws}^{TOT} > 0$ mJ m^{-2} they adhered to PS. The lack of agreement between thermodynamic and adhesion results proposes that bacterial adhesion on PS surfaces is not influenced by the surface physicochemical properties. *Sph. capsulata* physicochemical properties revealed the highest hydrophilicity, consequently, being the less prone to adhere to PS according to the thermodynamic approach. This bacterium had also the lowest ability to adhere to PS according to the adhesion assays. This demonstrates that the physicochemical properties account apparently for the low adhesion ability of *Sph. capsulata*. However, for the other bacteria, no correlation was found between cell surface hydrophobicity and their ability to adhere to PS. This fact is corroborated by other studies (Oliveira *et al.*, 2007; Sousa *et al.*, 2009), likely due to the multiplicity of parameters involved in the adhesion process being influenced both by biological and environmental factors. Also, it is perceptible that the zeta

potential differences do not influence the adhesion process. PS, *M. mucogenicum* and *Sph. capsulata* had highly negatively charged surfaces (zeta potential < -25 mV), while the other bacteria had surfaces with moderate negatively charged. However, there is no clear relationship between the zeta potential data and adhesion. Flint *et al.* (1997) were unable to assess any relationship between the numbers of *Streptococci* cells attaching to stainless steel and cell surface charge. Previous studies already reported the lack of a correlation between the bacterial surface properties and attachment. The attachment process was strongly influenced by the presence of extracellular biological molecules (Li and Logan, 2004; Chae *et al.*, 2006). Barton *et al.* (1996), however, found that surface growth of *Pseudomonas aeruginosa* on diverse polymers correlated with the free energy of adhesion, while no such correlation was found for *Staphylococcus epidermidis* and *Escherichia coli*. Simões *et al.* (2008b) found a correlation between the thermodynamic approaches and biofilm formation of a *Bacillus cereus* strain forming biofilms with low EPS content. In the current study, the lack of agreement between thermodynamic approaches and adhesion assays reinforces that biological mechanisms, such as the expression of extracellular appendages - adhesins that mediate specific interactions with substrata at a nanometer scale, during the irreversible phase of microbial adhesion, in addition to the physicochemical ones, are the plausible aspects mediating the entire adhesion process (Flint *et al.*, 1997; Doyle, 2000; Sinde and Carballo, 2000; Donlan, 2002; Rodrigues and Elimelech, 2009).

The importance of initial events in biofilm development still remains unknown due to the multitude of subsequent events taking place on a much longer time scale (Busscher and van der Mei, 1997). There are some evidences indicating that initial adhesion may be an important aspect in final biofilm formation, particularly for systems under fluctuating shear conditions (Quirynen *et al.*, 1993; Busscher and van der Mei, 1997). DWDS are usually subjected to variable hydraulic situations, ranging from no-flow (stagnant water) to steady-state hydrodynamic conditions. In this study, the magnitude of the initial bacterial adhesion on the subsequent biofilm formation was compared for the DW-isolated bacteria (under constant shear conditions) being found that only for *Methylobacterium* sp. and *M. mucogenicum*, both weakly adherent bacteria, are good biofilm producers regardless the biofilm age. Also, adhesion and biofilm formation are correlated when analyzing the 24 h aged biofilms. Non-adherent bacteria (*B. cepacia*, *Sph. capsulata* and *Staphylococcus* sp.) are non-biofilm producers or produce low biofilm amounts only for low aged biofilms (24 or 48 h). However, after a certain period of time all the bacteria had the ability to develop

biofilms. When increasing the biofilm formation period the relationship between adhesion and biofilm formation decreases. This time-dependent effects are evident when characterizing the *A. calcoaceticus* biofilms. This bacterium develops weak biofilms for a 24 h period, 24 h later (48 h aged biofilms) the biofilm formation ability decreases and 24 h (72 h aged biofilms) after the bacteria forms large biofilm amounts. This result indicates that the biofilm maturation process increases the system complexity and decreases the possibility of making reliable correlations with the early biofilm development stages. A recent report demonstrated the autoagregation ability of *A. calcoaceticus* (Simões *et al.*, 2008a). This bacterial ability provides an increased opportunity for metabolic cooperation in the early biofilm development process, being important not only for colonization, but also for biofilm development (Rickard *et al.*, 2003, 2004). Some authors (Fox *et al.*, 1990; Petrozzi *et al.*, 1993) already questioned the significance of the effect of the initial bacterial adhesion on biofilm formation because the number of bacterial cells involved in the initial biofilm formation process is much smaller than that in mature biofilms. However, other researchers have suggested that there is a link between the initially adhering bacteria and the biofilms that subsequently are formed (Busscher *et al.*, 1995). Motility is another important cellular aspect in the early stages of biofilm formation and development. Pratt and Kolter (1998) demonstrated that surface motility is an important factor in the initial interaction with an abiotic surface. Also, Kogure *et al.* (1998) have shown that motility increases adhesion to a bare glass substratum. This has been attributed to the increased collision frequency with the solid surface (Morisaki *et al.*, 1999). Comparing the current results with a previous study, it is evident that the motility of the tested DW isolates does not regulate adhesion and biofilm formation (Simões *et al.*, 2007b). *B. cepacia* has the highest motility, however, this bacterium is non-adherent and non- (24 h) or low biofilm producer (48 and 72 h). The remaining species had low motility values and similar between then (Simões *et al.*, 2007b). Roosjen *et al.* (2006) observed that the motility and zeta potential were not distinctive for adhesive and non-adhesive strains, and could therefore not be the reason for the difference in adhesion behaviour. In other study, no correlation between motility, adhesion and biofilm formation was found (Pompilio *et al.*, 2008). Also, those authors found a strong relationship between the extent of initial adhesion of *Stenotrophomonas maltophilia* to PS surfaces and biofilm formation.

In conclusion, controlling and preventing the adverse impact of the bacterial deposition on the aquatic environment needs an in-depth understanding about the

mechanisms regulating this process. The XDLVO theory has been used extensively to describe the deposition of bacteria in many current researches. However, physicochemical approaches based on the XDLVO theory were inappropriate for modelling adhesion of the tested DW bacteria to PS. The adhesion results suggest that mechanisms other than physicochemical surface properties may play a determinant role on bacterial adherence ability. Bacteria themselves produce extracellular molecules with sufficient surface activity to play a role in the bacterial adhesion process. However, the adhesion step does not provide conclusive information on the formation of mature biofilms. Adhesion ability was only correlated when comparing the results of the 24 h biofilms. Given time, all the bacteria had the ability to form biofilms even if considered non-adherent. *A. calcoaceticus*, *Methylobacterium* sp. and *M. mucogenicum* were classified as weakly adherent to PS and formed large biofilm amounts. The remaining bacteria were non-adherent; however, had the ability to form biofilms. This identification of the main bacteria forming more complex biofilms (*A. calcoaceticus*, *Methylobacterium* sp. and *M. mucogenicum*), probably more resistant to disinfection, due to their high biomass amount, may provide new information necessary for improving water quality for the consumers. Furthermore, these biofilms can act as a harbour and/or substrate for other microorganisms less prone to biofilm formation, increasing the probability of pathogen survival and further dissemination in the DWDS.

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CHAPTER 6 STUDY OF INTERGENERIC COAGREGATION BETWEEN DRINKING WATER BACTERIA

Intergeneric coaggregation of six DW autochthonous heterotrophic bacteria isolated from a model laboratory system were tested for its ability to coaggregate by a visual assay and by two microscopic techniques (epifluorescence and scanning electron microscopies). One isolate, identified as *Acinetobacter calcoacticus*, was found not only to autoaggregate, but also to coaggregate with four of the five other isolates (*Burkholderia cepacia, Methylobacterium sp., Mycobacterium mucogenicum, Sphingomonas capsulata* and *Staphylococcus sp.*) to different degrees as assessed by the visual assay, highlighting a possible bridging function in a biofilm consortium. In its absence, no coaggregation was found. Microscopic observations revealed a higher degree of interaction for all the aggregates than did the visual assay. Heat and protease reversed autoaggregation and coaggregation, suggesting that interactions were lectin-saccharide mediated. The increase/decrease in the level of extracellular proteins and polysaccharides produced during intergeneric bacteria association was not correlated with coaggregation occurrence, but probably with coaggregation strength. The putative bridging function of *A. calcoaceticus* was evidenced by multispecies biofilm studies, through a strain exclusion process.

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6.1 Introduction

Biofilm formation in DWDS improves DW contamination by reducing the microbiological safety through the increased survival of pathogens (Percival *et al.*, 1999; Tsai, 2005; Simões *et al.*, 2006). The knowledge of the main mechanisms promoting DW biofilm formation is of great interest as it can contribute to their understanding and control.

The development of microbial biofilm communities results from a series of processes, including initial surface association and adherence, subsequent multiplication of the constituent organisms, the adherence of other species and production of extracellular polymeric substances (Bryers, 2000). Many of these events leading to biofilm development, such as primary colonization, the expression of extracellular polymeric substances and gross phenotypic changes are well described (Stoodley *et al.*, 2002; Simões *et al.*, 2007c). The bacterial surface properties, coaggregation and coadhesion of bacteria and interspecies relationships are processes that are believed to play a determinant role in the formation of single and multispecies biofilms in DWDS (Rickard *et al.*, 2003b). Nevertheless, the function of coaggregation in the initial development of biofilm communities still remains unclear.

Coaggregation, the specific recognition and adherence of genetically distinct bacteria to one another, occurs in a variety of ecosystems (Malik *et al.*, 2003; Rickard *et al.*, 2003b) and was first demonstrated from bacteria from dental plaque (Gibbons and Nygaard, 1970). This adhesion mechanism is highly specific and is thought to have a role in the development of multispecies biofilms in many different environments (Kolenbrander and London, 1993; Kolenbrander *et al.*, 1999; Rickard *et al.*, 2003a) and now recognized as a mechanism that allows specific association between collaborating bacteria species. Aggregation conveys advantages to microorganisms. These include transfer of chemical signals, exchange of genetic information, protection from adverse environmental conditions, metabolic cooperation between different species as well as cell differentiation in some populations (Wimpenny and Colasanti, 2004). The coaggregation between pairs of bacteria is typically mediated by a protein “adhesin” on one cell type and a complementary saccharide “receptor” on the other. These protein-saccharide interactions could be blocked by the addition of simple sugars (Cisar *et al.*, 1979; Buswell *et al.*, 1997). Coaggregation interactions contribute to the development of biofilms via the specific recognition and adhesion of single suspended cells to genetically distinct bacteria in a developing biofilm

and/or by the subsequent adhesion of previously coaggregated secondary colonizers to the developing biofilm (Rickard *et al.*, 2003a). In both cases, bacterial cells in suspension specifically adhere to those within biofilms through a coadhesion process (Busscher *et al.*, 1995).

The purpose of the present work was to study the intergeneric coaggregation of six heterotrophic bacteria isolated from DW by visual coaggregation assay, SEM and epifluorescence microscopy. Extracellular proteins and polysaccharides (EPS) were assessed over time and correlated with coaggregation ability. The surface-associated molecules (proteins and saccharides) involved in coaggregation process were investigated by heat and protease treatment, and by sugar reversal tests. The role of *A. calcoaceticus* as bridging organism in DW biofilms was assessed by multispecies biofilms experiments, through a strain exclusion process.

6.2 Material and methods

6.2.1 Bacteria isolation and identification

The microorganisms used throughout this work were isolated from a model laboratory DWDS, as described previously in chapter 3 and by Simões *et al.* (2006). TSA (Merck, VWR, Portugal) and R2A (Oxoid, UK) were used for heterotrophic bacteria recovery and from DW and growth.

Bacteria were identified by 16S rDNA gene sequencing according to the method described in chapter 4 and by Simões *et al.* (2007a).

6.2.2 Bacterial cell growth and preparation of bacterial suspensions

The assays were performed with six representative DW isolated bacteria, *Acinetobacter calcoaceticus*, *Burkholderia cepacia*, *Methylobacterium* sp., *Mycobacterium mucogenicum*, *Sphingomonas capsulata* and *Staphylococcus* sp., respectively.

Bacterial cells were grown in batch culture using 200 ml of R_2A broth in 500 ml glass flasks (Schott, Duran), at room temperature (23 ºC ± 2), under agitation (150 rpm), until reaching the stationary growth phase as assessed by spectrometry (Spectronic 20 Genesys, Spectronic Instruments) at 640 nm. The stationary phase of growth was selected

because numerous works (Rickard *et al.*, 2002, 2003a, 2003b, 2004) refereed that coaggregation is growth-phase-dependent, being maximum when both partner bacteria are in stationary phase. Cells were harvested by centrifugation (20 min at 13000 g), washed three times in sterile tap water and resuspended in a certain volume of sterile tap water or R2A broth necessary to achieve the bacterial concentration needed for each assay.

6.2.3 Visual coaggregation assay

A visual coaggregation assay, with some modifications from the method of Cisar *et al.* (1979), was used to assess the ability of bacteria to coaggregate. Bacterial suspensions prepared as described above were resuspended in sterile tap water to an OD at 640 nm of 1.5 and mixed together in pairs by putting equal volumes (2 ml) of each cell suspension at room temperature in 10 ml rolled glass tubes. The mixtures were then vortexed for 10 s, and the tubes were rolled gently for 30 s. The degree of coaggregation between each pair was assessed visually in a semiquantitative assay, following the scoring scheme originally described by Cisar *et al.* (1979). If specific cell-to-cell recognition occurs, cells flocculate (coaggregate) and settle out. The scoring criteria were as follows: 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; 4, very large flocs of coaggregates that settle almost instantaneously, leaving a clear supernatant. Control tubes of each isolate on their own were also included to assess autoaggregation and scored by the same criteria. The coaggregation and autoaggregation scores were evaluated over time (0, 2, 24 and 48 h), staying this mixtures, during this period, at room temperature. Coaggregation was considered to be present when the score in the reaction mixtures was greater than the autoaggregation score of either strain.

6.2.4 Microscopy visualizations

Bacterial coaggregates were also observed (2 and 24 h) by epifluorescence microscopy using a DNA binding stain, DAPI, and by SEM. For epifluorescence microscopy visualizations, aliquots (15 µl) of bacterial autoaggregates and coaggregates were fixed using 2% (v v^{-1}) formaldehyde (Merck, Germany) and then filtrated through a 25

mm black Nuclepore® polycarbonate membrane with a pore size of 0.2 µm (Whatman, UK). After filtration, bacterial aggregates were stained with 100 µg ml^{-1} DAPI (Sigma) for 5 min and preparations were stored at 4 °C in the dark until visualization. Bacterial coaggregates were observed under an epifluorescence microscope (Carl Zeiss, Germany) equipped with a filter sensitive to DAPI fluorescence (359 nm excitation filter in combination with a 461 nm emission filter). Several microphotographs of the stained samples were obtained using a microscope camera (AxioCam HRC, Carl Zeiss) and a program path (AxioVision, Carl Zeiss Vision) involving image acquisition and image processing.

Prior to SEM observations, 100 µl of bacterial autoaggregates and coaggregates were fixed with 3% (v v^{-1}) glutaraldehyde (Riedel-de-Haën, Germany) in microtiter dishes (polystyrene, Orange Scientific, USA) for 1 h at room temperature and then 15 µl were placed in glass coverslips, dehydrated by heat (60 ºC, 2 h) and stored in a desiccator for 3 d. The samples were sputter-coated with gold and examined with a Leica S360 scanning electron microscope at 10-15 kV.

Microscopy visualizations were documented through the acquisition of at least 20 representative microphotographs.

6.2.5 Inhibition of coaggregation with simple sugars

The reversal or inhibition of coaggregation was determined by the addition of simple sugars: D(+) – galactose, N-acetyl-D-glucosamine, D(+) – fucose and D(+) – lactose (Sigma) to the bacterial coaggregating pairs. Filter-sterilized solutions of each simple sugar (500 mM in sterile deionised water) were added independently to coaggregating pairs to a final concentration of 50 mM. Mixtures were then vortexed and analyzed by the visual coaggregation assay. The inhibition or reversal of coaggregation was determined as a reduction in the coaggregation score.

6.2.6 Inhibition of coaggregation by heat treatment

The inhibition of coaggregation by heat pre-treatment of members of coaggregating pairs was performed using a method modified from that of Kolenbrander *et al.* (1985). Bacterial isolates suspensions were resuspended in sterile tap water to an OD at 640 nm of

1.5 and heated for 30 min at 80 ºC. Heat-treated and untreated bacterial cells were then combined in reciprocal pairs, and the capacity for the bacterial cells to coaggregate was assessed by the visual coaggregation assay. Inhibition or reversal of coaggregation was detected if coaggregation score decrease.

6.2.7 Inhibition of coaggregation by protease treatment

The protease sensitivity of the biopolymers mediating coaggregation on each element of the coaggregating pair was assessed using a modification of the method used by Cookson *et al.* (1995). Briefly, bacterial isolates suspension prepared as described above were resuspended to an OD at 640 nm of 1.5 in sterile tap water. Protease type XIV from *Streptomyces griseus* (P5147, Sigma) was added to the bacterial cell suspension to a final concentration of 2 mg ml^{-1}. Protease pre-treatment of bacteria was carried out at 37 ºC, and cells were harvested after 2 h by centrifuging and washing three times with sterile tap water. The bacterial suspensions were then readjusted to an OD at 640 nm of 1.5. Protease-treated and untreated cells were mixed, and their abilities to coaggregate were determined using the visual assay. Afterwards, inhibition or reversal of coaggregation was perceptible by coaggregation score decrease.

6.2.8 Extracellular proteins and polysaccharides extraction and quantification

Extraction of the EPS of the coaggregation partnerships was carried out (0 and 24 h) using Dowex resin (50X 8, NA^+ form, 20-50 mesh, Aldrich-Fluka 44445) according to the procedure described by Frølund *et al.* (1996). Prior to extraction, Dowex resin was washed with extraction buffer (2 mM Na_3PO_4, 2 mM NaH_2PO_4, 9 mM NaCl and 1mM KCl, pH 7). The suspensions containing the bacterial coaggregates were resuspended in 20 ml of extraction buffer and 50 g of Dowex resin per g of volatile solids were added to the coaggregation partnerships suspension and the extraction took place at 400 rpm for 4 h at 4 ºC. The extracellular components were separated from the cells through a centrifugation (13000 *g*, 20 min).

Total proteins were determined using the Lowry modified method (SIGMA-Protein Kit nº P5656) using bovine serum albumin as standard and total polysaccharides content by the phenol-sulphuric acid method of Dubois *et al.* (1956) using glucose as standard.

6.2.9 Multispecies biofilm formation in microtiter plates

Multispecies biofilm formation was performed with all the representative DW bacteria used in this study. Thus, biofilms were developed at seven different bacterial combinations, one mixture of all six bacteria and six combinations with a mixture of five distinct bacteria, through a strain exclusion process (biofilm formation in the absence of a specific strain, obtaining distinct species combinations).

Biofilms were developed according to the modified microtiter plate test proposed by Stepanović *et al.* (2000) using R2A broth as growth medium. For each condition at least 16 wells of a sterile 96-well flat tissue culture plates (polystyrene, Orange Scientific, USA) were filled under aseptic conditions with 200 µl of a cell suspension mixture (10^8 cells ml^{-1}). Biofilms were developed with equal initial cell densities of each isolate. To promote biofilm formation, plates were incubated aerobically on an orbital shaker, at 150 rpm and room temperature, for 24, 48 and 72 h. The growth medium was discarded and freshly added every 24 h. Negative controls were obtained by incubating the wells with R2A broth without adding any bacterial cells.

After each biofilm formation period, the content of each well was removed and the wells were washed three times with 250 µl of sterile distilled water to remove non-adherent and weakly adherent bacteria. The plates were air dried for 30 min, and the remaining attached bacteria were analysed in terms of the amount of biomass adhered on the microtiter plates surfaces using CV stain according to Simões *et al.* (2007b). The relative biofilm formation percentage was assessed by comparing biofilms formed by the strain exclusion process relative to biofilms formed by the mixture of all strains. All experiments were performed in triplicate, with three repeats.

6.2.10 Statistical analysis

The data were analysed using the statistical program SPSS version 14.0. Because low samples numbers contributed to uneven variation, total proteins and polysaccharides and adhesion results were analyzed by the nonparametric Wilcoxon test. Statistical calculations were based on a confidence level of $\geq$ 95% ($P < 0.05$ was considered statistically significant).

6.3 Results

6.3.1 Visual coaggregation ability of drinking water bacteria

Six numerically dominant heterotrophic bacteria (*A. calcoaceticus*, *B. cepacia*, *Methylobacterium* sp., *M. mucogenicum*, *Sph. capsulata*, *Staphylococcus* sp.) isolated from tap water coming from a DWDS in the Braga, Portugal were identified by 16S ribosomal DNA gene sequencing and their coaggregation partnerships were determined immediately after dual bacteria mixture, 2, 24 and 48 h later, using a visual coaggregation assay (Table 6.1).

Table 6.1 Coaggregation scores over time of DW bacteria by the visual assay

Time (h) of assay for *A. calcoaceticus* and indicated partner strain	**Coaggregation scores for bacterium[a]**					
	Sph. capsulata	*B. cepacia*	*M. mucogenicum*	*Methylobacterium* sp.	*A. calcoaceticus*	*Staphylococcus* sp.
0	**2/3**	**3**	**2/3**	1/2	2	**3/4**
2	**3/4**	**2/3**	**3**	2	2	**3/4**
24	**4**	2	**3**	2	2	**3/4**
48	**4**	2	**3**	2	2	**3/4**

[a]Bold numbers indicate the bacterial interactions with effective coaggregation. Values separated by slashes indicate an intermediate value between the two scores.

A. calcoaceticus coaggregated with four of the five other bacteria, the exception being *Methylobacterium* sp. (Table 6.1). The other bacteria did not coaggregate in the absence *A. calcoaceticus*. Coaggregation, after immediate bacteria association, was higher for *A. calcoaceticus* with *Staphylococcus* sp. (coaggregation score of 3/4), with an invariable score throughout the 48 h of the experiment. *A. calcoaceticus*/*B. cepacia* was the only interaction that decreased the coaggregation score after incubation. All other interactions increased (*A. calcoaceticus*/*Methylobacterium* sp., *A. calcoaceticus*/*M. mucogenium*, *A. calcoaceticus*/*Sph. capsulata*) coaggregation scores over time. Comparing coaggregation scores over time, it is shown that the maximum score was achieved 24 h after bacteria-bacteria contact, as this value was similar 48 h after. Analyzing the coaggregation scores after 24 or 48 h using a score increasing factor it is found the following order: *A. calcoaceticus*/*B. cepacia* = *A. calcoaceticus*/*Methylobacterium* sp. < *A. calcoaceticus*/*M.*

mucogenicum < *A. calcoaceticus*/*Staphylococcus* sp. < *A. calcoaceticus*/*Sph. capsulata. A. calcoaceticus*/*Sph. capsulata* is the coaggregation partnership with the highest time-coaggregation score increment.

Autoaggregation experiments revealed that only *A. calcoaceticus* had autoaggregation ability, with easily visible small aggregates (score 2), being this score constant along the experiment. The other bacteria did not autoaggregate (Table 6.1).

6.3.2 Coaggregation detection by microscopic methods

The coaggregates were observed over time (2 and 24 h – time required for maximum aggregation scores) by epifluorescence microscopy using DAPI and by SEM. Figure 6.1 shows several representative microphotographs concerning various interactions between the distinct DW bacteria with and without visual coaggregation. Microscopic analysis revealed a higher degree of interaction than did the visual coaggregation assay. This feature was evident for all the interactions, even for autoaggregation.

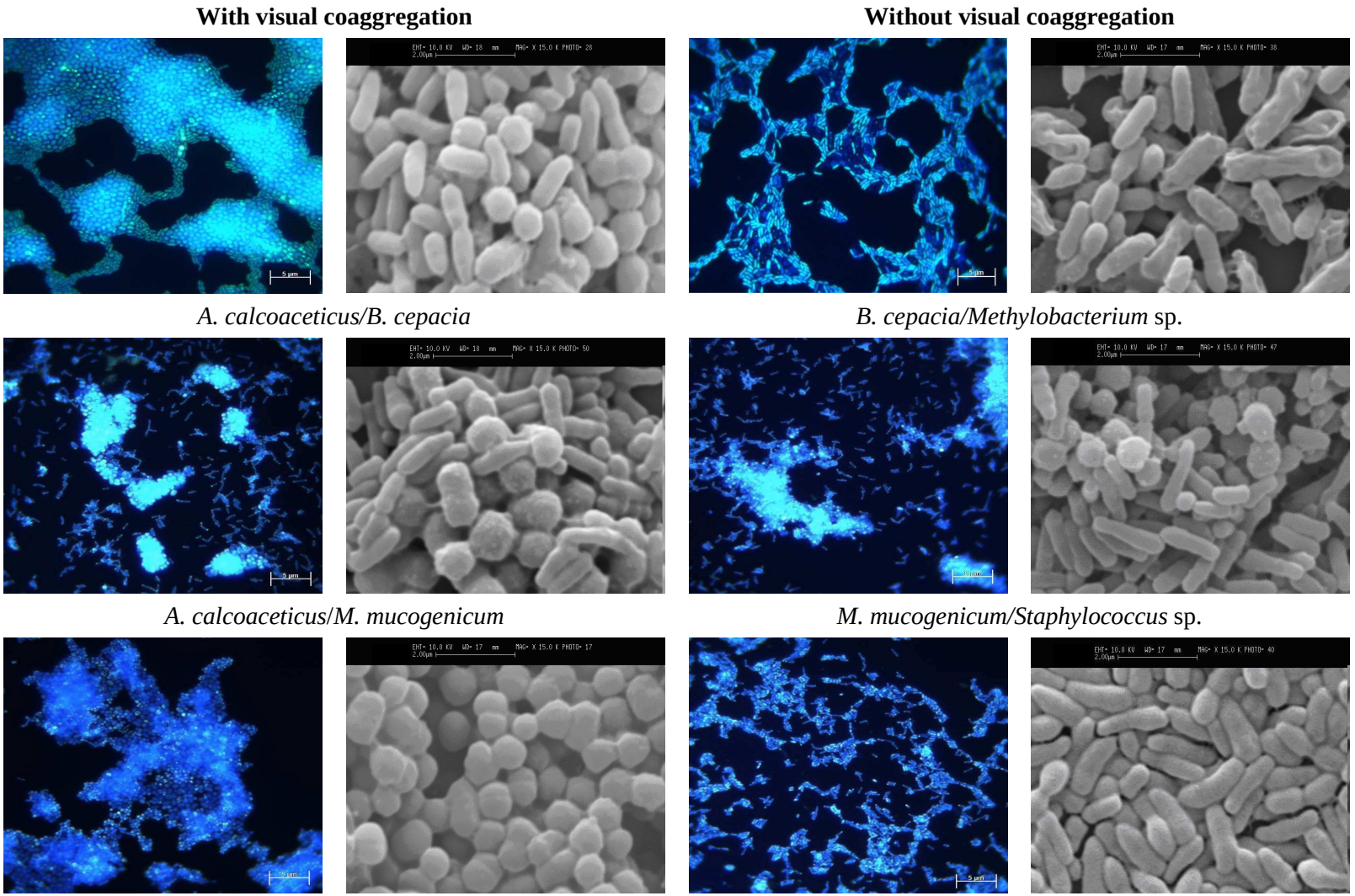


Figure 6.1 Microscopy visualizations by epifluorescence microscopy and SEM of the distinct interacting DW bacteria with and without visual coaggregation.

Magnification, ×1320; bar = 5 μm (epifluorescence photomicrographs). Magnification, ×8000; bar = 5 μm (SEM photomicrographs).

6.3.3 Effect of simple sugars, heat and protease treatment on coaggregation

In order to determine the surface-associated molecules involved in coaggregation several inhibition assays were performed, by the addition of simple sugars, heat and protease treatment. Inhibition or reversal of coaggregation was determined as a reduction in the coaggregation score.

Coaggregation between several pairs of bacteria was inhibited by the addition of some simple sugars (Table 6.2).

Table 6.2 Reversal of coaggregation using simple sugars

A. calcoaceticus **coaggregation partner**	**Results for sugars[a]**			
	D (+) - Galactose	**N-Acetyl-D-Glucosamine**	**D(+) - Fucose**	**D(+) - Lactose**
Sph. capsulata	+	–	+	+
B. cepacia	–	–	–	–
M. mucogenicum	–	–	–	–
Methylobacterium sp.	+	–	+	+
A. calcoaceticus	–	–	–	–
Staphylococcus sp.	–	–	–	–

[a]++ Complete disaggregation; + partial disaggregation; - no disaggregation.

N-acetyl-D-glucosamine was the only simple sugar unable of reverse any coaggregation partnership. The others sugars reversed two coaggregating pairs, *A. calcoaceticus*/*Sph. capsulata* and *A. calcoaceticus*/*Methylobacterium* sp. For the other situations no disaggregation was detected. The simple sugars only caused a partial disaggregation, not able to completely reverse the coaggregation (score 0). No autoaggregation (*A. calcoaceticus*/*A. calcoaceticus*) inhibition was detected by simple sugars (Table 6.2).

Table 6.3 shows the effect of heat and protease treatment on coaggregation scores when each partner was pretreated separately by those two kinds of treatments and then mixed with either a treated or an untreated partner. Heat and protease treatment, when applied to both partners, led to complete coaggregation inhibition (score 0) for all coaggregation partnerships, except that of *A. calcoaceticus*/*Sph. capsulate*, with a partial

inhibition for heat treatment (score 1). When only one partner was treated, and if it was *A. calcoaceticus*, the results were similar to those observed when both partners were treated, except for protease treatment were it was only observed almost complete inhibition for *A. calcoaceticus*/*Sph. capsulata* and *A. calcoaceticus*/*M. mucogenicum* (score 0/1). No disaggregation was verified if the treated partner was one of the other bacteria, except *A. calcoaceticus/Sph. capsulata* and *A. calcoaceticus*/*M. mucogenicum* (heat and protease) and *A. calcoaceticus/B. cepacia* (protease). For these cases, the interaction score decreased lightly, translated in a partial disaggregation. Protease treatment was more efficient than heat treatment on coaggregation inhibition of *A. calcoaceticus*/*Sph. capsulata* and *A. calcoaceticus*/*B. cepacia*. For other coaggregation partnerships the same results were obtained with the two treatments.

Analyzing the effect of heat and protease treatment on autoaggregation, it was verified *A. calcoaceticus* autoaggregation inhibition. No inhibition was detected when treated cells were mixed 1:1 with untreated cells (Table 6.3).

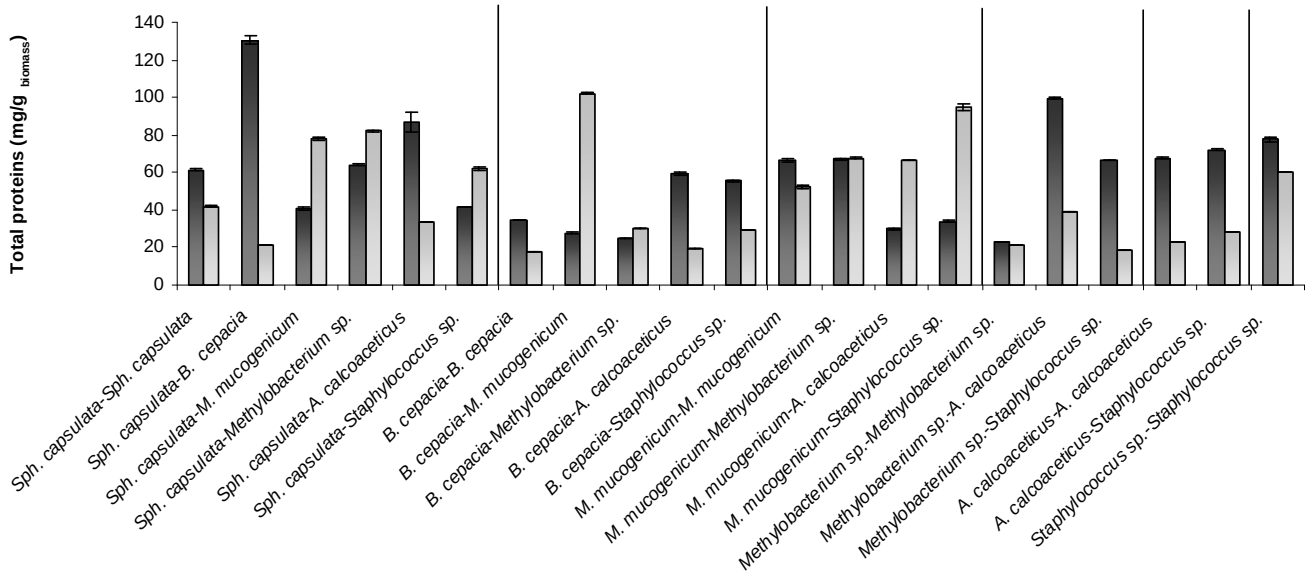
Table 6.3 The effect of heat and protease treatment on coaggregation scores[a]

***A. calcoaceticus* treatment type**	**Coaggregation scores for bacterium with indicated partner type[b]**											
	Sph. capsulata		*B. cepacia*		*M. mucogenicum*		*Methylobacterium* sp.		*A. calcoaceticus*		*Staphylococcus* sp.	
	UT	T	UT	T	UT	T	UT	T	UT	T	UT	T
Heat												
UT	4	3	2	2	3	2	2	2	2	2	3/4	3/4
T	1	1	0	0	0	0	0	0	2	0	0	0
Protease												
UT	4	2	2	1/2	3	2	2	2	2	2	3/4	3/4
T	0/1	0	0	0	0/1	0	0	0	2	0	0	0

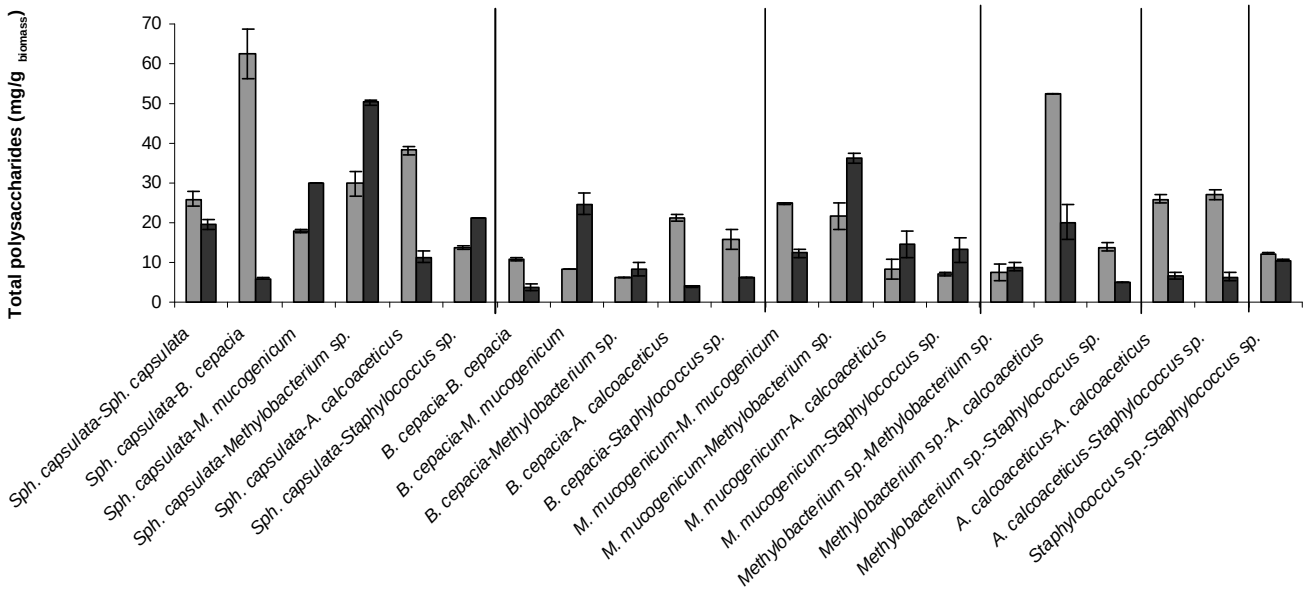
[a]Scores shown are results for when each partner was pretreated separately with heat and protease and then mixed with either a treated (T) or an untreated (UT) partner.
[b]Values separated by slashes indicated an intermediate value between the two scores.

6.3.4 Extracellular proteins and polysaccharides production by bacterial aggregates

With the aim to correlate the EPS production with coaggregation ability of DW bacteria, it was assessed their production over time (0 and 24 h) for all coaggregation partnerships (Figure 6.2).



a)



b)

Figure 6.2 Extracellular proteins (a) and polysaccharides (b) of the coaggregation partnerships over time, 0 h (□) and 24 h (■). The means ± standard deviations (error bars) for at least three replicates are illustrated.

An increase on total proteins content was found for *Sph. capsulata*/*M. mucogenicum*, *Sph. capsulata*/*Methylobacterium* sp., *Sph. capsulata*/*Staphylococcus* sp., *B. cepacia*/*M. mucogenicum*, *B. cepacia*/*Methylobacterium* sp., *A. calcoaceticus*/*M. mucogenicum*, *M. mucogenicum*/*Staphylococcus* sp. (Figure 6.2a). Maintenance on proteins content was found for *M. mucogenicum*/*Methylobacterium* sp. and *Methylobacterium* sp. autoaggregates. Statistical equivalence ($P > 0.05$) of proteins level for the two sampling time was found for

B. cepacia/*Methylobacterium* sp., *M. mucogenicum*/*Methylobacterium* sp., and *Methylobacterium* sp./*Methylobacterium* sp. For the other interactions the proteins content decreased over time. Regarding over time variation of total polysaccharides content, Figure 6.2b shows that an increase on polysaccharides content occurs for the interactions between *Sph. capsulata*/*M. mucogenicum*, *Sph. capsulata*/*Methylobacterium* sp., *Sph. capsulata*/*Staphylococcus* sp., *B. cepacia*/*M. mucogenium*, *B. cepacia*/*Methylobacterium* sp., *M. mucogenicum*/*Methylobacterium* sp., *A. calcoaceticus*/*M. mucogenicum*, *M. mucogenicum*/*Staphylococcus* sp., *A. calcoaceticus*/*Methylobacterium* sp., *Methylobacterium* sp./*Staphylococcus* sp., *A. calcoaceticus*/*Staphylococcus* sp. and *A. calcoaceticus* autoaggregates. Maintenance on polysaccharides content was found for *Methylobacterium* sp. and *Staphylococcus* sp. autoaggregates. For the other situations the polysaccharides content decreased over time. Statistical equivalence ($P > 0.05$) on polysaccharides level for the two sampling times were found for *B. cepacia*/*Methylobacterium* sp., *Methylobacterium* sp./*Methylobacterium* sp., and *Staphylococcus* sp./*Staphylococcus* sp. Thus, the number of the coaggregation partnerships with reduction on the total extracellular proteins and polyssacharides content over time is far more than that increasing. All coaggregation partnership had a similar over time variation in total proteins and polyssacharides ($P>0.05$).

6.3.5 Multispecies species biofilm formation in microtiter plates

In order to ascertain the putative bridging function of *A. calcoaceticus* in DW bacterial interactions, mixed biofilm formation was carried out with the six isolates. Figure 6.3 shows that all tested combinations formed biofilms on microtiter plates. Biofilm mass increased over time, except for multispecies biofilms without *A. calcoaceticus*. Only those without *Methylobacterium* sp. had similar biomass amounts for the three sampling times ($P > 0.05$). The combination excluding *M. mucogenicum* formed the highest biofilm mass ($P < 0.05$). Bacterial combinations without *A. calcoaceticus* exhibited the smallest productivity for 48 and 72 h ($P < 0.05$), while at 24 h it was the bacterial combination without *B. cepacia*.

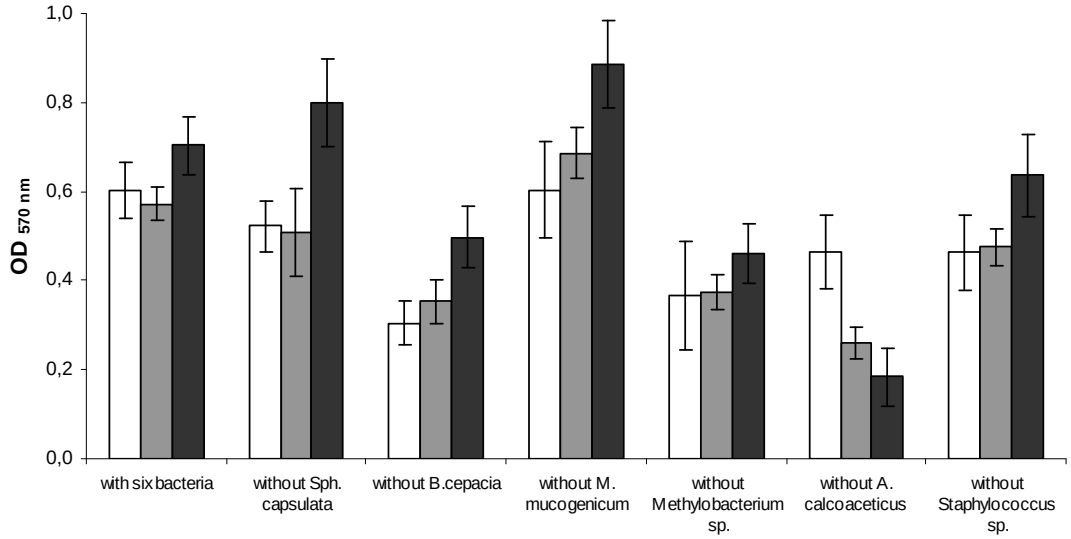


Figure 6.3 Values of OD at 570 nm ($OD_{570\ nm}$) as a measure of multispecies biofilm mass for 24 h (□), 48 h (▒) and 72 h (■). The means ± standard deviations (error bars) for at least three replicates are illustrated.

To better understand the function of each bacterium in multispecies biofilm formation, the relative percentage of biofilm formation by the combination of all six DW bacteria was assessed in the strain exclusion tests and compared with the multispecies biofilms formed by the six bacteria (Table 6.4).

Table 6.4 Relative multispecies biofilms formation over time

Bacteria present in multispecies biofilm[a]	**Relative(%) biofilm formation at indicated time (h)**		
	24	**48**	**72**
All six bacteria	100	100	100
All except:			
S*ph. capsulata*	86.6	88.8	114
B. cepacia	50.6	61.7	70.7
M. mucogenicum	100	120	126
Methylobacterium sp.	60.5	65.2	65.3
A. calcoaceticus	76.8	45.3	26.1
Staphylococcus sp.	76.8	83.0	90.5

[a]Members of the group of six bacteria studied were *Sph. capsulata*, *B. cepacia*, *M. mucogenicum*, *Methylobacterium* sp., *A. calcoacteticus* and *Staphylococcus* sp.

M. mucogenicum was the only bacterium that when not present led to a relative increase of biofilm mass over time compared to the level of biofilm formation with all six bacteria. The remaining bacteria reduced biofilm formation. The decrease of biofilm mass formation was less significant ($P > 0.05$) for biofilms in the absence of *Sph. capsulata* (24 and 48 h) and *Staphylococcus* sp. (72 h), and more significant ($P < 0.05$) in the absence of *B. cepacia* (24 h) and *A. calcoaceticus* (48 and 72 h). Nevertheless, even if the relative biofilm formation decreased for five of the six strain exclusion scenarios, it was only significant ($P < 0.05$) and decreased over time ($P < 0.05$) for biofilms without *A. calcoaceticus*.

6.4 Discussion

Biofilm formation and development is a consequence of several types of cell-cell interactions between different pairs and groups of bacteria (Buswell *et al.*, 1997). Coaggregation has been pointed as one of the main mechanisms of adhesion that can enhance the potential for bacterial biofilm development (Rickard *et al.*, 2003a). This adhesion mechanism is highly specific and is thought to have a role in the development of multispecies biofilms in many diverse environments (Malik *et al.*, 2003; Rickard *et al.*, 2003a). Consequently, it is important to study the involvement of coaggregation in multispecies bacterial formation and behaviour. As suggested and demonstrated by Rickard *et al.* (2003b), coaggregation is a phenomenon that occurs most frequently between bacteria from natural multispecies biofilm than from planktonic population. This phenomenon is likely to enhance the development of freshwater multispecies biofilms and may influence biofilm species diversity in the natural environment (Rickard *et al.*, 2003b). In this study, six heterotrophic DW isolated bacteria (Simões *et al.*, 2007a), belonging to different genera, were analyzed in terms of coaggregation ability over time by a visual assay and by microscopic techniques. Coaggregation was detected between several pairs of the autochthonous water flora. However, this phenomenon had the particularity of occurring only in the presence of *A. calcoaceticus* as assessed by the visual assay. The other bacteria did not coaggregate in its absence. Moreover, *A. calcoaceticus* was also the only tested bacteria with the ability to autoaggregate.

Microscopy analyses revealed some degree of interaction, not detected by the visual evaluation. According to Buswell *et al.* (1997), low visual coaggregation scores are not necessarily indicators of weak interaction between cells. The scores detected with this assay are not accurate measures of the relative interaction strength between individual ligands on different cells. Furthermore, these authors proposed that visual coaggregation will depend on the relative sizes and morphologies of the bacteria involved and may depend on the densities of interacting ligands on the bacterial surface. A lack of sensitivity associated with the visual assay was also proposed by Elliott *et al.* (2006). Nevertheless, the rapid and simple visual assay provided reproducible results with enough sensitivity to detect significant interactions (Buswell *et al.*, 1997).

Coaggregation is a highly specific process involving interactions between bacterial surface molecules that act as adhesins and complementary receptors, including proteins and carbohydrates. In most of the situations, heat and protease treatments of coaggregating pairs, totally inhibited coaggregation. The interactions between the tested coaggregation partnerships are apparently mediated by heat- and protease-sensitive adhesins of *A. calcoaceticus* and heat- and protease-stable interactive sites on the surface of the other bacterium. However, for *A. calcoaceticus* with *Sph. capsulata* and *A. calcoaceticus* with *M. mucogenicum*, the results suggests the existence of other type of interactions between heat- and protease-stable receptors in *A. calcoaceticus* and heat- and protease-sensitive adhesins in *Sph. capsulata* and *M. mucogenicum*. Heat and protease treatment inhibited *A. calcoaceticus* autoaggregation. However, no inhibition was detected when treated cells were mixed 1:1 with untreated cells. This result demonstrates not only those heat- and protease-sensitive proteins (lectins) mediate aggregation between the tested bacteria, but also those other molecules, such as saccharides, that can bind to lectins of untreated cells may be involved. Moreover, this result also suggests that *A. calcoaceticus* extracellular binding molecules are apparently constituted by lectins and saccharides, therefore increasing the interaction potential with other bacteria (Rickard *et al.*, 2003a). In fact, many bacteria have been found to possess proteinaceous adhesins on their surfaces that bind, in a stereochemically specific manner, to complementary molecules/receptors (often saccharides) on the surfaces of other bacterial cells of the same or different species (Skillman *et al.*, 1999; Rickard *et al.*, 2003a). The ability of simple sugars to reverse the coaggregation process was not verified for all coaggregating bacterial pairs. For those with reversed coaggregation, interactions were only partially inhibited. The addition of simple

sugars was expected to reverse the lectin-saccharide (protein-carbohydrate)-like interactions. Nevertheless, such interactions are known to be very specific (Kolenbrander *et al.*, 1989). It is possible that neither the selected sugars nor the tested concentrations were appropriate. Kolenbrander *et al.* (1995) found that depending upon the involved bacterial pairs, a varied response to the addition of sugar was observed in the case of potential lectin-saccharide-like coaggregation of oral pathogens. Other authors also found that protein-carbohydrate-like interaction between *Candida albicans* and *Actinomyces* species was not reversed by sugars (Grimaudo *et al.*, 1996). A study by Malik *et al.* (2003) shows that, reversibility by simple sugars is not an essential feature of lectin-like interactions. Although the present study could not elucidate the exact nature of the surface molecules involved in coaggregation, the results suggest the possibility of lectin-saccharide-like interactions involvement. This finding is in agreement with the previous studies of Rickard *et al.* (2002, 2003b) about the interactions mediating coaggregation on freshwater bacteria belonging generally to different species from the ones used in this study.

The exact function of EPS, which are secreted by microorganisms during growth, are not completely elucidated because of their extremely heterogeneous nature. It has been reported that EPS may play a significant role in the formation and function of microbial aggregate, including matrix structure formation and microbial physiological processes (Tsuneda *et al.*, 2003; Teschke, 2005). The increased production of EPS is an important early physiological event that can occur during the development of a biofilm, and which might be important in the adhesion of secondary colonizers. These polymers envelop the attached cells within the biofilm, strengthen their adhesion and can act as receptors for coaggregation interactions (Rickard *et al.*, 2003a). In this study, it was found that, in some cases, the amount of proteins and polysaccharides increased over time. It is tempting to speculate that the bacterial community organization may be explained on the basis of weak and strong cell-to-cell interactions combined with bacterial metabolic and chemotactic properties. In some SEM inspections it is also perceptible that EPS-like structures were present in the intercellular spaces. However, besides coaggregation being lectin and saccharin dependent, it was not correlated with EPS content. The over time variation of the extracellular proteins and polysaccharides content seemed not to account for the coaggregation phenomenon, but could be arguably involved in the coaggregation strength and not in their occurrence. Further studies, such as those based on atomic force

microscopy, are required in order to provide more evidences on the role of EPS level on coaggregation.

Under natural environments, monospecies biofilms are rare. Conversely, microorganisms are associated as complex multispecies sessile communities. Diversity in microbial communities leads to a variety of complex relationships involving interspecies and intraspecies interactions which can influence profoundly biofilm formation and development (Skillman *et al.*, 1999). Multispecies biofilms formed by the isolated bacteria were performed in order to identify the role of *A. calcoaceticus* as bridging microorganism between this DW microflora. In fact, *A. calcoaceticus* coaggregated with the other DW bacteria tested, suggesting the ability to form multigeneric coaggregates and a potential bridging function, in a manner similar to those of *Fusobacterium* sp. and *Prevotella* sp. in dental plaque accretion (Kolenbrander *et al.*, 1985; Kolenbrander, 1989). Rickard *et al.* (2002) reported similar findings for *Blastomonas natatoria* in freshwater bacteria communities. An *A. johnsonii* strain has also been proposed as bridging bacterium in an activated sludge microflora (Malik *et al.*, 2003). Such bridging microorganisms are believed to carry complementary receptors recognized by functionally similar adhesins on cells from distinct genera (Malik *et al.*, 2003). The role of *A. calcoaceticus* as bridging bacteria is reinforced by studies of strain exclusion from the multispecies biofilms. Biofilm formation decreased for five of the six strain exclusion scenarios. Nevertheless, it was only significant and decreased over time for biofilms without *A. calcoaceticus*. This result provides additional evidence concerning the role of *A. calcoaceticus* in DW microbial ecosystems.

In conclusion, to our knowledge this is the first report demonstrating that *A. calcoaceticus* performs a bridging function in DW biofilm formation. This bacterium coaggregates with almost all other tested bacteria, and its presence in a multispecies community represents a colonization advantage. This bacterium may facilitate the association of the other species that do not coaggregate directly with each other, increasing the opportunity for metabolic cooperation. The presence or the absence of *A. calcoaceticus* in multispecies biofilms can therefore enhance or decrease, respectively, biofilm formation by DW bacteria.

Acknowledgments

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CHAPTER 7 BACTERIAL INTERACTIONS IN DRINKING WATER BIOFILMS

In the environment, multiple microorganisms coexist as communities, competing for resources and often associated as biofilms. In this study, single and dual species biofilm formation by, and specific activities of, six heterotrophic intergeneric bacteria, was determined using 96-wells polystyrene plates over a 72 h period. These bacteria were isolated from DW and identified by partial 16s rRNA gene sequencing. A series of planktonic studies were also performed, assessing the bacterial growth rate, motility and production of QSI. This constituted an attempt to identify key attributes allowing bacteria to effectively interact and coexist in a DW environment. We observed that in both pure and dual cultures, all the isolates formed stable biofilms within 72 h, with specific metabolic activity decreasing, in most cases, with an increase in biofilm mass. The largest single and dual biofilm amounts were found for *Methylobacterium* sp. and the combination of *Methylobacterium* sp. and *Mycobacterium mucogenicum*, respectively. Evidences of microbial interactions in dual biofilms formation, associated with appreciable biomass variation in comparison with single biofilms, were found for the following cases: synergy/cooperation between *Sphingomonas capsulata* and *Burkholderia cepacia*, *Sph. capsulata* and *Staphylococcus* sp., and *B. cepacia and Acinetobacter calcoaceticus*; antagonism between *Sph. capsulata and M. mucogenicum*, *Sph. capsulate and A. calcoaceticus,* and *M. mucogenicum-Staphylococcus* sp. A neutral interaction was found for *Methylobacterium* sp.-*M. mucogenicum*, *Sph. capsulata-Staphylococcus* sp, *M. mucogenicum-A. calcoaceticus* and *Methylobacterium* sp.-*A. calcoaceticus* biofilms, since the resultant dual biofilms had a mass and specific metabolic activity similar to the average of each single biofilm. *B. cepacia* had the highest growth rate and motility, and produced QSI. Other bacteria producing QSI were *Methylobacterium* sp., *Sph. capsulata* and *Staphylococcus* sp. However, only for *Sph. capsulata-M. mucogenicum*, *Sph. capsulata-A. calcoaceticus* and *M. mucogenicum-Staphylococcus* sp., dual biofilm formation seems to be regulated by the QSI produced by *Sph. capsulata* and *Staphylococcus* sp. and by the increased growth rate of *Sph. capsulata*. The parameters assessed by planktonic studies did not allow prediction and generalization of the exact mechanism regulating dual species biofilm formation between the DW bacteria.

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7.1 Introduction

DW systems are known to harbour biofilms, even though these environments are oligotrophic and often contain a disinfectant. Control of these biofilms is important for aesthetic and regulatory reasons (Percival and Walker, 1999; Volk and LeChevallier, 1999; Tsai, 2005). The interaction of pathogens with existing biofilms has predominantly been a concern with man-made water systems, particularly DWDS (Block, 1992; Percival and Walker, 1999; Szewzyk *et al.*, 2000). Microorganisms are generally less a problem in planktonic phase due to the increased susceptibility to disinfection (Simões *et al.*, 2003, 2005). The examination of a DWDS reveals the complexity of such a technical system. There are not only many different materials used for the transportation and regulation of the water flow, but also dramatic variations in the flow conditions between different locations (Berry *et al.*, 2006; Simões *et al.*, 2007a). Obviously, microorganisms face a diversity of habitats with distinct physicochemical and nutritional conditions during treatment, storage, and distribution of DW (Szewzyk *et al.*, 2000; Simões *et al.*, 2007a). Bacteria are affected not only by the environment they live in, but also by the variety of other species present. By performing studies on the interactions present in multispecies biofilms, basic knowledge on several aspects of sociomicrobiology can be gained (Parsek and Greenberg, 2005; Burmølle *et al.*, 2006). A range of interactions have been observed among microorganisms in biofilms, including antagonistic, mutualistic, competitive and commensal relationships (Burgess *et al.*, 1999; Cowan *et al.*, 2000; Nielsen *et al.*, 2000; Christensen *et al.*, 2002; Tait and Sutherland, 2002; Rao *et al.*, 2005; Burmølle *et al.*, 2006). For instance, competition amongst microorganisms, for space and nutrients, is a powerful selective force which has led to the evolution of a variety of effective strategies for colonising and growing on surfaces (Burgess *et al.*, 1999; Szewzyk *et al.*, 2000). The mechanisms that control microbial interactions, in multispecies biofilms, are not yet fully understood (Szewzyk *et al.*, 2000; Komlos *et al.*, 2005). The ecology of a biofilm is a complex equation of physicochemical and biological parameters. As with all levels of evolution, a complex web of interactions is central to the structure, composition and function of these or any communities (Hansen *et al.*, 2007). Optimizing the management of DWDS and controlling microbial growth are difficult due to the complexity of these systems. The study of bacterial ecology and ethology might help to improve our understanding of the persistence of biofilms and associated pathogens, in DWDS. There is evidence that biofilm community

diversity can affect disinfection efficacy and allow pathogens to survive within biofilms (Elvers *et al.*, 2002; Burmølle *et al.*, 2006). The assessment of microbial mechanisms, regulating multispecies biofilm formation, becomes a very important tool for the determination of the composition of DW bacteria, because they likely lead to the predominance of the best adapted species, for that set of conditions. The knowledge of biofilm biodiversities and its species physiology may facilitate the development of DW disinfection and biofilm control processes.

The aim of this study was to assess the role of interspecies interactions in dual species biofilm formation and characteristics. Furthermore, we sought to assess possible key factors (growth rate, motility and production of QS antagonists) regulating microbial interactions between intergeneric DW bacteria.

7.2 Material and methods

7.2.1 Bacteria isolation and identification

The microorganisms used throughout this work were isolated from a model laboratory DWDS, as described previously in chapter 3 and by Simões *et al.* (2006). Briefly, two consecutive GAC filter columns were directly plugged into the normal tap water from the Braga (Portugal) water distribution network. The first GAC filter eliminated the free chlorine contained in the tap water, while the second was a biological activated filter furnishing a continuous bacterial inoculum to a Perspex chemostat (volume, 1.6 l; diameter, 16.8 cm). The system was sterile until filled with potable water and operated so as to prevent immigration of microorganisms other than via the tap water feeding. The flow rate of tap water gave a dilution rate of 3.125 h^{-1}. Microorganisms were isolated by collecting 100 µl of the chemostat water and plating on both TSA (Merck, VWR, Portugal) and R2A (Oxoid, UK) aerobically at room temperature (23 ± 2 ºC) for 15 d. This two media were already tested successfully in the recovery of heterotrophic bacteria from DW (Reasoner and Geldrich, 1985; Simões *et al.*, 2006).

Preliminary, presumptive bacteria identification was done using selective medium Chromocult® TBX agar (Merck), *Pseudomonas* isolation agar (Difco), Metanol minimum medium, according to the method of Kim *et al.* (1999), Gram-staining and biochemical

methods (API 20 NE and API ID32 GN systems (Biomerieux)) according to the manufacturer's instructions. Further identification tests for determination of the 16S rRNA gene sequence were performed for putative bacteria, according to the procedure described in chapter 4 and by Simões *et al.* (2007a).

7.2.2 Planktonic bacterial growth

Assays were performed with six representative DW bacteria, *Acinetobacter calcoaceticus* (031), *Burkholderia cepacia* (010), *Methylobacterium sp.* (029), *Mycobacterium mucogenicum* (017), *Sphingomonas capsulata* (003) and *Staphylococcus sp.* (052). The bacterial genera used in this study represented more than 80% of the total genera isolated and identified.

Bacterial cells were grown overnight in batch culture using 100 ml of R2A broth, at room temperature, under agitation (150 rpm). Cells were harvested by centrifugation (20 min at 13000 g), washed three times in 0.1 M of PBS (KH_2PO_4; Na_2HPO_4, NaCl) and resuspended in a certain volume of R2A broth necessary to achieve a cellular density of 10^8 cells ml^{-1}.

7.2.3 Biofilm formation in microtiter plates

Biofilms were developed according to the modified microtiter plate test proposed by Stepanović *et al.* (2000). Briefly, for each bacterium at least 16 wells of a sterile 96-well flat tissue culture plates (polystyrene, Orange Scientific, USA) were filled under aseptic conditions with 200 µl of cell suspension (10^8 cells ml^{-1} in R2A broth). To promote biofilm formation, the plates were incubated aerobically on a shaker at 150 rpm, at room temperature for 24, 48 and 72 h. Every 24 h, the growth medium was carefully discarded and replaced by a fresh one. At each sampling time, the content of each well was removed and washed three times, with 250 µl of sterile distilled water, to remove non-adherent and weak adherent bacteria. The plates were air dried for 30 min, with the remaining attached bacteria being analysed in terms of biomass adhered on the inner walls of the wells, and in terms of their respiratory activity. Negative controls were obtained by incubating the wells only with R2A broth without adding any bacterial cells. All experiments were performed in triplicate with three repeats.

7.2.4 Mass quantification by CV

The bacterial biofilms in the 96-wells plates were fixed with 250 μl $well^{-1}$ of 98% methanol (Vaz Pereira, Portugal), for 15 min. Afterwards, the plates were emptied and left to dry. Then, the fixed bacteria were stained for 5 min with 200 μl $well^{-1}$ of CV (Gram-colour-staining set for microscopy, Merck). Excess stain was rinsed out by placing the plate under low running tap water (Stepanović *et al.*, 2000). After the plates were air dried, the dye bound to the adherent cells was resolubilized by 200 μl $well^{-1}$ of 33% (v v^{-1}) glacial acetic acid (Merck, Portugal). The OD of the obtained solution was measured at 570 nm using a microtiter plate reader (BIO-TEK, Model Synergy HT) and biofilm mass was presented as $OD_{570\ nm}$ values.

Bacteria were classified using the scheme of Stepanović *et al.* (2000) as follows: non-biofilm producer (0): $OD \leq OD_c$; weak biofilm producer (+): $OD_c < OD \leq 2 \times OD_c$; moderate biofilm producer (++): $2 \times OD_c < OD \leq 4 \times OD_c$; strong biofilm producer (+++): $4 \times OD_c < OD$. This classification was based upon the cut-off OD (OD_c) value, defined as three standard deviation values above the mean $OD_{570\ nm}$ of the negative control.

7.2.5 Activity assessment by XTT staining

The sodium 3,3'-[1[(phenylamino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) colorimetric method was applied to determine the bacterial activity of the biofilms as described previously by Stevens and Olsen (1993), with some modifications. Briefly, 200 μl of a combined solution of XTT (Sigma) and phenazine methosulfate (PMS) (Sigma) was added to each well, in order to obtain a final concentration of 50 μg ml^{-1} of XTT and 10 μg ml^{-1} PMS. Then, the microtiter plates were incubated for 3 h and 150 rpm, at room temperature, in the dark. The OD of the formazan supernatant of each well was measured at 490 nm using a microtiter plate reader (BIO-TEK, Model Synergy HT). The biofilm specific respiratory activity was presented as $OD_{490\ nm/570\ nm}$ (biofilm respiratory activity/biofilm mass).

7.2.6 Bacterial screening for QSI and AHL production

Test bacteria were streaked on the centers of R2A agar plates and grown overnight at room temperature. Indicator microorganisms were grown overnight in LB broth

(*Chromobacterium violaceum* O26 and *C. violaceum* 12472) or LB plus 50 µg ml^{-1} spectinomycin (Sigma) and 4.5 µg ml^{-1} tetracycline (*Agrobacterium tumefaciens* A136). Following overnight growth, the test bacteria were overlaid with 5 ml LB soft agar (full strength LB broth containing 0.5% w v^{-1} agar), cooled to 45 °C, containing 10^6 CFU ml^{-1} of the indicator microorganisms *C. violaceum* ATCC 12472. *P. aeruginosa* PAO-1 was used as a positive control for QSI, since its two signal molecules, 3-oxo-dodecanoyl homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl homoserine lactone (C4-HSL), competitively bind and inhibit the receptor for the cognate signal *N*-hexanoyl homoserine lactone (C6-HSL), in both indicator microorganisms. *C. violaceum* ATCC 12472 was used as a negative control, since it produces the cognate C6-HSL and would therefore not inhibit its own QS signal. A positive QSI result was indicated by a lack of pigmentation of the indicator microorganism, in the vicinity of the test microorganism.

A bioassay for AHL production was performed in order to detect the type of molecule responsible for QSI (McLean *et al.*, 2004). Two biosensor microorganisms, *A. tumefaciens* A136 and *C. violaceum* CVO26, that directly respond to AHLs were used. The *A. tumefaciens* biosensor is highly sensitive to a variety of AHL chains, ranging from C6 to C14, while *C. violaceum* is unable to synthesize its endogenous C6-HSL inducer, but retains the ability to respond to C4-HSL and C6-HSL. For the bioassay, test bacteria were grown on R2A as described above. Following overnight growth, the bacteria were overlaid with LB soft agar containing 5 µl of overnight cultures of *C. violaceum* CVO26 or the *A. tumefaciens* A136 biosensor and incubated overnight at 30 ºC. Following incubation, 50 µl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Sigma) solution (20 mg ml^{-1} in dimethylformamide) was added to the *A. tumefaciens* A136 assay plates and colour development, due to X-Gal hydrolysis, allowed to proceed for 15 min at room temperature. *A. tumefaciens* KYC6, a 3-oxo C8 HSL overproducer, was used as positive control for the *A. tumefaciens* biosensor. *C. violaceum* 31532 was the positive control for the *C. violaceum* CVO26 assay. The biosensor strains themselves were used as negative controls, since both strains lack AHL synthase genes. A positive test for AHLs was indicated by a blue coloration from X-Gal hydrolysis, in the *A. tumefaciens* biosensor, or by a purple CVO26 pigmentation. Negative tests for AHLs were indicated by a lack of coloration (McLean *et al.*, 2004).

7.2.7 Motility assays

R2A broth overnight cultures were used to assay motility in plates containing 1% tryptone, 0.25% NaCl and 0.3% agar. The motility halos were measured at 8, 16, 24 and 48 h (Sperandio *et al.*, 2002). Three plates were used to evaluate each bacterium motility, experiments were conducted with two independent cultures.

7.2.8 Statistical analysis

The data were analysed using the statistical program SPSS, version 14.0. The mean and standard deviation within samples were calculated for all cases. Because low sample numbers contributed to uneven variation, the nonparametric Wilcoxon test was used to compare biofilm characteristics. Statistical calculations were based on a confidence level equal or higher than 95% (a P value of < 0.05 was considered statistically significant).

7.3 Results

7.3.1 Single species biofilm formation and specific respiratory activity

In order to assess the biofilm formation ability and specific respiratory activity of the several bacteria isolated from DW, the standard 96-wells microtiter plates with CV and XTT staining were used to characterize biofilms (Figure 7.1). Figure 7.1a shows that the tested bacteria formed biofilms, with *Methylobacterium* sp. producing the largest biomass amount, for all the sampling times. *M. mucogenicum* was the second stronger biofilm producer. A directly proportional time - biomass formation relationship was found for the various bacteria ($P < 0.05$), except for *B. cepacia* ($P > 0.05$). *Sph. capsulata* showed biofilm formation ability only for sampling times greater than 48 h. The biofilm amount was statistically similar only between *A. calcoaceticus* and *Staphylococcus* sp., when results for the various sampling times were compared ($P > 0.1$).

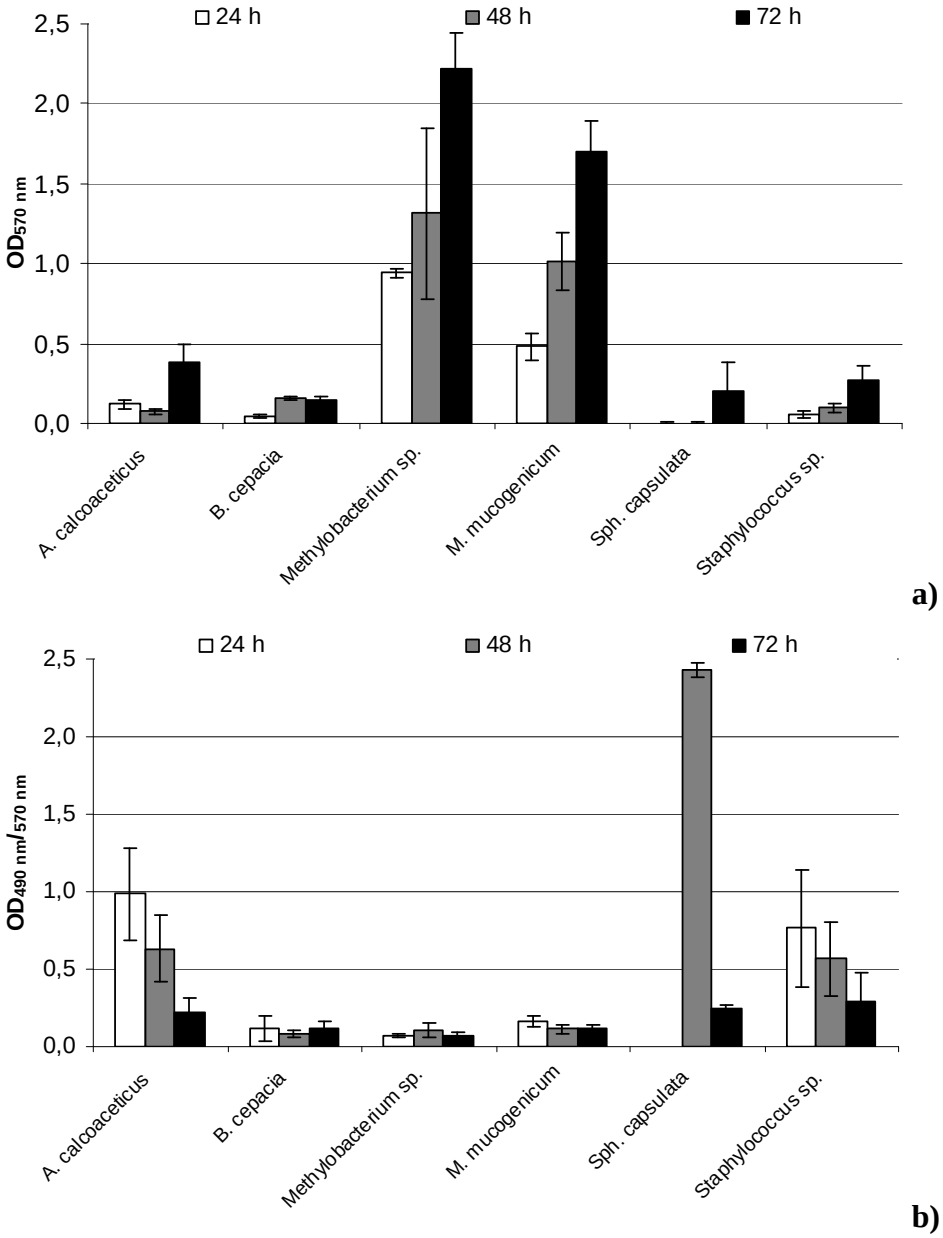

Figure 7.1 $OD_{570\ nm}$ and $OD_{490\ nm/570\ nm}$ values as a measure of single species biofilm mass (a) and specific respiratory activity (b) for DW bacteria. The means ± SDs for at least three replicates are illustrated.

Figure 7.1b shows that *B. cepacia*, *Methylobacterium* sp. and *M. mucogenicum* biofilms maintained their specific respiratory activities with similar values for the three bacteria ($P > 0.1$). *A. calcoaceticus* and *Staphylococcus* sp. formed biofilms with similar specific respiratory activities ($P > 0.05$), but their values decreased along time ($P < 0.05$). *Sph. capsulata* formed small biomass amounts (Figure 7.1a), with an $OD_{570\ nm}$ that was always smaller than 0.5. However, 48 h aged biofilms had the highest specific respiratory activities ($OD_{490\ nm/570\ nm}$ higher than 2.3) compared with those of the other bacteria. *Sph.*

capsulata specific respiratory activity from the 48 h biofilms sharply decreased, 1 d after, for $OD_{490\ nm/570\ nm}$ values smaller than 0.5 (Figure 7.1b).

A rank of biofilm formation was produced according to the method of Stepanović *et al.* (2000), classifying test bacteria as non-biofilm producer, weak biofilm producer, moderate biofilm producer or strong biofilm producer (Table 7.1).

Table 7.1 Biofilm formation abilities of DW isolated bacteria[a]

Bacterium	Biofilm formation at sampling time (h)		
	24	48	72
A. calcoaceticus (031)	+	0	+++
B. cepacia (010)	0	+	+
Methylobacterium sp. (029)	+++	+++	+++
M. mucogenicum (017)	+++	+++	+++
Sph. capsulata (003)	0	0	++
Staphylococcus sp. (052)	0	0	++

[a]According to the classification proposed by Stepanović *et al.* (2000): (0) non-biofilm producer; (+) weak biofilm producer; (++) moderate biofilm producer; (+++) strong biofilm producer.

Methylobacterium sp. and *M. mucogenicum* showed a strong biofilm producing ability, for the various sampling times. *Sph. capsulata* and *Staphylococcus* sp. presented biofilm formation ability (moderate) only for the 72 h sampling time. *B. cepacia* formed weak biofilms after 48 h, while *A. calcoaceticus* showed variability in biofilm formation ability by forming weak biofilms at 24 h, being classified as a non-biofilm producer at 48 h, and as a strong biofilm producer at the 72 h sampling time.

7.3.2 Dual species biofilm formation and specific respiratory activity

Dual species biofilms studies showed time dependent biofilm formation ability (Figure 7.2a), with a statistical level of significance ($P < 0.05$) for the following biofilms: *Sph. capsulata-B. cepacia* (003-010), *Sph. capsulata-Methylobacterium* sp. (003-029), *B. cepacia-M. mucogenicum* (010-017), *B. cepacia-Methylobacterium* sp. (010-029), *B. cepacia-A. calcoaceticus* (010-031), *B. cepacia-Staphylococcus* sp. (010-052), *M. mucogenicum-Methylobacterium* sp. (017-029) and *M. mucogenicum-A. calcoaceticus* (017-031). Dual *Sph. capsulata-A. calcoaceticus* (003-031) and *Sph. capsulata-Staphylococcus*

sp. (003-052) biofilms decreased in mass over time ($P < 0.05$). The remaining biofilms maintained a stable biomass over time, since biofilm mass differences for the different sampling times did not reach a level of statistical significance ($P > 0.05$).

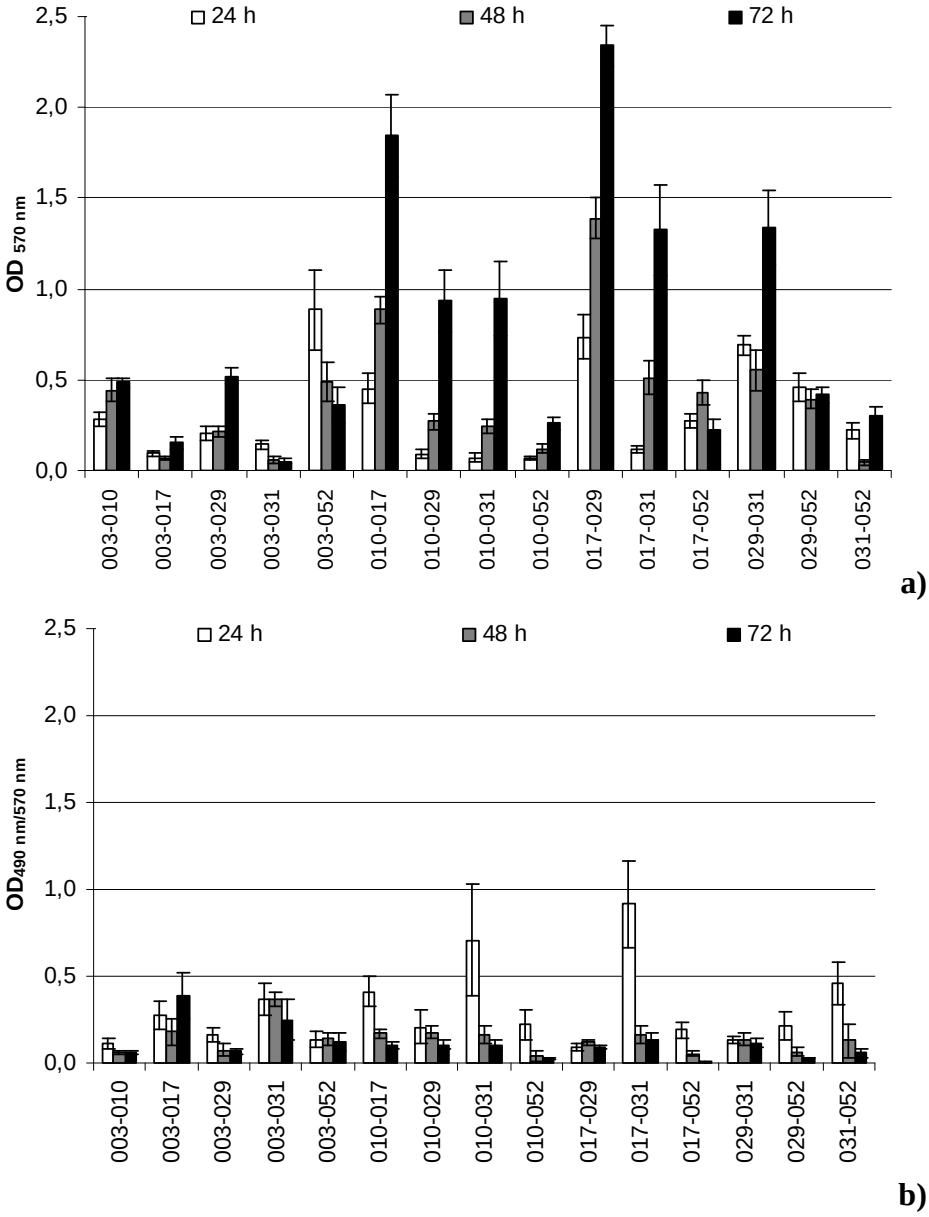

Figure 7.2 $OD_{570\ nm}$ and $OD_{490\ nm/570\ nm}$ values as a measure of dual species biofilm mass (a) and specific respiratory activity (b) for DW bacteria. The means ± SDs for at least three replicates are illustrated.

XTT biofilm reaction for specific biofilm respiratory activity assessment showed that for 11 of the 15 dual biofilms studied (Figure 7.2b), a statistically significant ($P < 0.05$) decrease of the $OD_{490\ nm/570\ nm}$ value occurred over time. This was found for the following dual biofilms: *Sph. capsulata-B. cepacia* (003-010), *Sph. capsulata-Methylobacterium* sp.

(003-029), *Sph. capsulata-A. calcoaceticus* (003-031), *B. cepacia-M. mucogenicum* (010-017), *B. cepacia-Methylobacterium* sp (010-029), *B. cepacia-A. calcoaceticus* (010-031), *B. cepacia-Staphylococcus* sp. (010-052), *M. mucogenicum-A. calcoaceticus* (017-031), *M. mucogenicum-Staphylococcus* sp. (017-052), *Methylobacterium* sp.-*Staphylococcus* sp. (029-052) and *A. calcoaceticus-Staphylococcus* sp. (031-052). The other biofilms maintained statistical similar specific respiratory activity values, for the several sampling times ($P > 0.05$).

As for the single biofilms (Table 7.1), an equivalent ranking was attributed for dual biofilms (Table 7.2).

Table 7.2 Biofilm formation ability of dual species of bacteria[a]

Interaction[b]	Biofilm formation at sampling time (h)		
	24	48	72
003 - 010	++	++	++
003 – 017	0	0	+
003 – 029	+	+	+++
003 – 031	+	0	0
003 – 052	+++	++	++
010 - 017	+++	+++	+++
010 – 029	0	++	+++
010 – 031	0	++	+++
010 – 052	0	0	++
017 – 029	+++	+++	+++
017 – 031	+	+++	+++
017 – 052	++	++	+
029 – 031	+++	+++	+++
029 – 052	++	++	++
031 - 052	+	0	++

[a]According to the classification proposed by Stepanović *et al.* (2000): (0) non-biofilm producer; (+) weak biofilm producer; (++) moderate biofilm producer; (+++) strong biofilm producer

[b]Bacterial species are as follow: 003, *Sph. capsulata*; 010, *B. cepacia*; 017, *M. mucogenicum*; 029, *Methylobacterium* sp.; 031, *A. calcoaceticus*; 052, *Staphylococcus* sp.

This ranking showed that *B. cepacia-M. mucogenicum*, *M. mucogenicum-Methylobacterium* sp., and *Methylobacterium* sp.-*A. calcoaceticus* interactions produced strong biofilms for the three sampling times. Strong biofilms were also found at 24 h for

Sph. capsulata-Staphylococcus sp. interactions, at 48 h for *M. mucogenicum-A. calcoaceticus* and at 72 h for *Sph. capsulata-Methylobacterium* sp., *B. cepacia-Methylobacterium* sp., *B. cepacia-A. calcoaceticus* and *M. mucogenicum-A. calcoaceticus*. *Sph. capsulata-B. cepacia* and *Methylobacterium* sp.-*Staphylococcus* sp. formed moderate biofilms at the various sampling times. Other bacterial interactions producing moderate biofilms were found for *Sph. capsulata-Staphylococcus* sp. at 48 and 72 h, *B. cepacia-Methylobacterium* sp. at 48 h, *B. cepacia-A. calcoaceticus* at 48 h, *B. cepacia-Staphylococcus* sp. at 72 h, *M. mucogenicum-Staphylococcus* sp. at 24 and 48h and *A. calcoaceticus-Staphylococcus* sp. at 72 h sampling time. Weak biofilm production was found for *Sph. capsulata-M. mucogenicum* interactions at 72 h, *Sph. capsulata-Methylobacterium* sp. at 24 and 48 h, *Sph. capsulata-A. calcoaceticus* at 24 h, *M. mucogenicum-A. calcoaceticus* at 24 h, *M. mucogenicum-Staphylococcus* sp. at 72 h and *A. calcoaceticus-Staphylococcus* sp. at 24 h. The remaining microbial interactions/sampling times drew the non-biofilm producer classification.

The comparisons between single and dual biofilms showed the existence of interspecies microbial interactions. Biofilm interspecies relationships were based on the comparison between dual species biofilms characteristics (ranking) and those from each single biofilm. The existence of synergistic or antagonistic interactions in dual biofilm formation was considered whether the biofilm formation category of each single bacterium (Table 7.1) was lesser or greater, respectively, than that found for dual biofilms (Table 7.2). Accordingly, evident antagonistic interactions were found for 72 h aged biofilms of *Sph. capsulata-M. mucogenicum*, *Sph. capsulata-A. calcoaceticus* and *M. mucogenicum-Staphylococcus* sp. Cooperation in biofilm formation, increasing biomass, was found for *Sph. capsulata-B. cepacia* and *Sph. capsulata-Staphylococcus* sp. both 24 and 48 h aged biofilms and for *B. cepacia-A. calcoaceticus* (48 h). Neutral interactions was found for *Methylobacterium* sp.-*M. mucogenicum* for all sampling times, and 72 h aged biofilms of *Sph. capsulata-Staphylococcus* sp, *M. mucogenicum-A. calcoaceticus* and *Methylobacterium* sp.-*A. calcoaceticus*, since the resultant biofilms had biomass and specific metabolic activity similar to the average of each ancestral biofilm.

7.3.3 Bacterial growth rate, motility, and production of QSI and AHL

To determine which factors influenced the interaction of the several bacteria in a dual species biofilm, a series of planktonic experiments was performed. Planktonic studies were performed with the test bacteria to assess bacterial growth rate in R2A broth (Table 7.3); motility (Table 7.4); and production of QSI and AHLs (Table 7.5), according to the methodology described by McLean *et al.* (2004). The referred parameters were evaluated in order to assess their role in dual species biofilm formation and activity.

Table 7.3 Bacterial planktonic growth rates

Bacterium	Growth rate (h^{-1})
A. calcoaceticus	0.0313 ± 0.008
B. cepacia	0.174 ± 0.005
Methylobacterium sp.	0.112 ± 0.004
M. mucogenicum	0.0757 ± 0.015
Sph. capsulata	0.119 ± 0.003
Staphylococcus sp.	0.0893 ± 0.014

According to the results presented in Table 7.3, *B. cepacia* showed the highest growth rate and *A. calcoaceticus* the lowest. *Methylobacterium* sp. and *Sph. capsulata* had similar growth rates ($P > 0.05$), which were higher than those of *M. mucogenicum* and *Staphylococcus* sp. ($P < 0.05$).

Table 7.4 Assessment of bacterial motility

Bacterium	Motility (cm) at sampling time (h)			
	8	16	24	48
A. calcoaceticus	0.5	1.0	1.0	1.5
B. cepacia	1.5	7.5	9.5	48.5
Methylobacterium sp.	1.0	1.0	1.0	1.5
M. mucogenicum	0.5	1.0	1.0	1.0
Sph. capsulata	0.5	0.5	1.0	1.5
Staphylococcus sp.	1.0	1.0	1.0	1.0

Table 7.4 shows motility results of the test bacteria. All presented an increase in motility over time, from 8 to 48 h, except *Staphylococcus* sp. This bacterium had an

invariable motility for the various times. A significantly time-increased motility was verified for *B. cepacia*, the bacterium with the highest motility values for all of the sampling times. The remaining species had very close motility values.

Studies of QSI screening (Table 7.5) showed that *B. cepacia*, *Methylobacterium* sp., *Staphylococcus* sp. and *Sph. capsulata* produced QSI molecules. Only for *Staphylococcus* sp. QSI were not related to AHLs. This was expected due to the peptide-like molecules involved in Gram-positive bacterium QS events not detected by the methodology used.

Table 7.5 Screening for QSI and AHL molecules[a]

Bacterium	QSI	Presence of AHLs	
		(C4-HSL and C6-HSL)	(C6-HSL-C14-HSL)
A. calcoaceticus	-	-	-
B. cepacia	+	+	+
Methylobacterium sp.	+	+	+
M. mucogenicum	-	-	-
Sph. capsulata	+	-	+
Staphylococcus sp.	+	-	-

[a]-, not detected; +, detected.

7.4 Discussion

An understanding of the microbial ecology of distribution systems is necessary to design innovative and effective control strategies that will ensure safe and high-quality DW. Recent investigations into the microbial ecology of DWDS have found that pathogen resistance to chlorination is affected by the community biodiversity and interspecies relationships (Berry *et al.*, 2006). In this study, some of the bacterial isolates tested (*B. cepacia*, *M. mucogenicum* and *Staphylococcus* sp.) are recognized as problematic opportunistic bacteria (Zanetti *et al.*, 2000; Conway *et al.*, 2002; Rickard *et al.*, 2004; Stelma *et al.*, 2004). The selected bacterial species were detected in DW biofilms. In fact, biofilms on surfaces exposed to DW, in distribution systems, may well be the main source of planktonic bacteria since up to 1000 sessile microorganisms can be present for each planktonic cell detected (Momba *et al.*, 2000). Microbial growth control is a key issue in fulfilling DW quality standards. All the isolated bacteria, belonging to distinct genera, had

the ability to form biofilms (Figure 7.1a) during the 72 h study. In some particular cases (*A. calcoaceticus*, *Staphylococcus* sp.), the specific metabolic activity (Figure 7.1b) was determined to be inversely related to the biofilm mass increase. In fact, following microbial attachment, the formation of a complex extracellular polymeric matrix increased the non-metabolically active biofilm mass (Simões *et al.*, 2003, 2005, 2007b), consequently decreasing the specific respiratory activity ($OD_{490\ nm/570\ nm}$). *Methylobacterium* sp. and *M. mucogenicum* held the greatest biofilm formation ability, while *A. calcoaceticus*, *B. cepacia*, *Sph. capsulata* and *Staphylococcus* sp. produced small biofilm mass amounts, with an $OD_{570\ nm} < 0.5$ (Figure 7.1a). This fact is also sustained by the biofilm formation ability score shown in Table 7.1. Contradicting these single species biofilm results, in a previous study (Simões *et al.*, 2007a), assessing bacterial adherence to selected polymeric surfaces, it was found that only *A. calcoaceticus* was strongly adherent to DW-related materials while only *Staphylococcus* sp. was moderately adherent. This allows the speculation that adhesion ability results do not necessarily predict the best biofilm forming bacteria and final biofilm characteristics, a fact also observed by other authors (Heilmann *et al.*, 1996, 1997).

Under natural conditions, true monospecies biofilms are rare, occurring mostly as complex communities. Because multispecies interactions prevail in the environment, dual biofilm studies were carried out but with the experimental conditions used for single biofilms. The dual biofilm approach was an attempt to get closer to the reality of naturally occurring biofilms. Although still being a great simplification of true biofilms, not fully replicating reality, it is still a more exact approach. The physiology and metabolism of multispecies biofilm communities are immensely complex (Rickard *et al.*, 2003). Diversity in microbial communities leads to a variety of complex relationships involving inter and intraspecies interactions (Berry *et al.*, 2006). The specific mechanisms for multispecies biofilm formation and organization still remain unclear. Nevertheless, a more complete picture of microbial community diversity and interspecies relationships should facilitate and contribute to the understanding of biofilm formation process and persistence in DW systems and other systems. From an ecological point of view, both competition and cooperation can exist in DWDS (Szewzyk *et al.*, 2000). In a recent study (Hansen *et al.*, 2007) concerning biofilm interspecies interactions, it was found that a derived dual species (*Acinetobacter* sp. and *Pseudomonas putida*) community was more stable and productive than the ancestral community. Previously, Møller *et al.* (1998) revealed a metabolic synergy between *P. putida* and *Acinetobacter* sp. community members for the biodegradation of toluene and

related aromatic compounds. Cooperative interactions between bacteria have been demonstrated mainly for degradation processes (Møller *et al.*, 1998; Cowan *et al.*, 2000; Szewzyk *et al.*, 2000). According to Burmølle *et al.* (2006), beneficial interactions in multispecies biofilms can include coaggregation and plasmid conjugation, contributing to the protection of one or several species from eradication even when the biofilm is exposed to external stress factors. In low-nutrient environments, such as DW, it may be assumed that oligocarbophilic-obligate bacteria are the first colonizers of the system, and that copiotrophs are second colonizers, using remnants and excretions of the original colonizers (Szewzyk *et al.*, 2000).

Most research into interspecies interactions within biofilms has focused on the beneficial aspects of these relationships. However, not all interactions will be advantageous for the several interacting microorganisms. Antagonistic interactions may play an important role in the development and structure of microbial communities. Competition for substrate is considered to be one of the major evolutionary driving forces in the bacterial world, and numerous experimental data obtained in the laboratory under well-controlled conditions show how different microorganisms may effectively outcompete others as a result of a better utilization of a given energy source (Christensen *et al.*, 2002; Komlos *et al.*, 2005; Rao *et al.*, 2005). The production of antagonistic compounds also seems to be a common phenomenon for some bacteria (Tait and Sutherland, 2002; Rao *et al.*, 2005; Bhattarai *et al.*, 2006). The present results show that the association of bacteria forming larger single biofilm mass amounts also formed dual biofilms (*M. mucogenicum-Methylobacterium* sp.) with the highest $OD_{570\ nm,}$ for the differently aged biofilms (Figure 7.2a). Other bacterial interactions/associations producing considerable biofilm amounts over time were found for *B. cepacia-M. mucogenicum* and *Methylobacterium sp.-A. calcoaceticus*. Cooperation in biofilm mass development does not necessarily lead to a highest specific respiratory activity. For the most significant situations, specific metabolic activity was inversely proportional to biofilm mass formation, a fact even more evident than for single species biofilms (Figure 7.2b). Stabilization of the interaction between the various dual species biofilms is likely to have implications not only for the community composition, structure and activity but also for the interspecies relationships and function of the community. Evidences of intraspecies relationships was assessed by the analysis of the differential biomass formation of single (Table 7.1) and dual (Table 7.2) biofilms. By this approach, mutualism/synergy in biofilm formation was found with the association of *Sph. capsulata-B.*

cepacia, *Sph. capsulata-Staphylococcus* sp. and *B. cepacia-A. calcoaceticus*, a fact probably related to the establishment of intergeneric metabolic cooperation. Competition/antagonistic interactions allowing the formation of smaller biofilm amounts were found for the following situations: *M. mucogenicum-Staphylococcus* sp., *Sph. capsulata-M. mucogenicum* and *Sph. capsulata-A. calcoaceticus* (Table 7.2). Neutral interaction was apparently existent for *Methylobacterium* sp.-*M. mucogenicum*, *Sph. capsulata-Staphylococcus* sp, *M. mucogenicum-A. calcoaceticus* and *Methylobacterium* sp.-*A. calcoaceticus* biofilms, since the resultant coculture had phenotypic characteristics similar to the average of each single biofilm (Figures 7.1 and 7.2).

The mechanisms that control biofilm microbial interactions in the environment are not fully understood (Rickard *et al.*, 2003; Komlos *et al.*, 2005). The existence of multiple interactions or even the simple production of a metabolite can interfere with the development of what seems to be structurally organized communities existing within a biofilm. To determine which factors may influence the interaction of the studied dual species biofilms, a series of batch experiments was performed, allowing the assessment of the bacterial growth rate, motility, QSI and AHLs production. These features are recognized as important factors regulating biofilm formation and interspecies interactions (Pratt and Kolter, 1998; Harshey, 2003; Daniels *et al.*, 2004; McLean *et al.*, 2004; Komlos *et al.*, 2005; Moons *et al.*, 2006). According to existing descriptions of multispecies biofilm population dynamics (Wanner and Gujer, 1986; Banks and Bryers, 1991), the faster growing bacteria should outcompete those that grow more slowly. QS, a cell density-related communication mode between one or more species, is a significant factor adding complexity to the interactions between biofilm bacteria. It is also known to influence bacterial community development in aquatic biofilms (McLean *et al.*, 2005). QS plays a role in cell attachment and detachment from biofilms (Davies *et al.*, 1998; Donlan, 2002; Daniels *et al.*, 2004). It involves an environmental sensing system that allows bacteria to monitor and respond to their own population densities. The bacteria produce a diffusible organic signal, generally called an autoinducer molecule, differing from Gram-negative to Gram-positive bacteria. It accumulates in the bacterial surrounding environment during growth (Fuqua and Greenberg, 2002). QS systems are known to be involved in a range of important microbial activities. These include extracellular enzyme biosynthesis, biofilm development, antibiotic biosynthesis, biosurfactant production, extracellular polymeric substances synthesis and extracellular virulence factors in Gram-negative bacteria (Passador *et al.*, 1993; Beck *et al.*,

1995; Chatterjee *et al.*, 1995; Pearson *et al.*, 1995; Davies *et al.*, 1998; Daniels *et al.*, 2004). In a dual biofilm, interference with this system through QSI of one bacterial species to another will arguably influence the proportion of each species in the biofilm. Motility is known to be essential for biofilm formation, overcoming the electrostatic repulsion of cells and surfaces (Pratt and Kolter, 1998; Harshey, 2003). High bacterial motility constitutes a surface colonization advantage (Harshey, 2003). Predictive biofilm bacterium dominance results, assessed by planktonic tests (Tables 7.3 to 7.5), shows that *B. cepacia* had the highest growth rate, motility and produced QSI (AHL-related molecules). It was apparently the bacteria with the best competitive advantage in cocultured biofilms. *Methylobacterium* sp. and *Sph. capsulata* also presented considerable high growth rate and motilities compared with the remaining bacteria, being also QSI producers. However, none of the bacteria showing competitive advantage in terms of an increased growth rate (*B. cepacia*, *Methylobacterium* sp. and *Sph. capsulata*), motility (*B. cepacia*) or QSI production (*B. cepacia*, *Methylobacterium* sp., *Sph. capsulata* and *Staphylococcus* sp.) conferred their specific characteristics to dual biofilms. In fact, antagonistic interactions in biofilm formation were only found for the *Sph. capsulata-M. mucogenicum*, *Sp. capsulata-A. calcoaceticus* and *M. mucogenicum-Staphylococcus* sp. dual biofilms. For these situations, biofilm formation seems to be regulated by the QSI produced by *Sph. capsulata* and *Staphylococcus* sp. and by the increased growth rate of *Sph. capsulata*. Moreover, in these same cases, planktonic tests demonstrate the inefficiency of the tested parameters in predicting microbial interactions in mixed biofilms, since dual biofilms formed by bacteria with a competitive advantage, *i.e.*, *Sph. capsulata-B. cepacia* and *Sph. capsulata-Staphylococcus* sp., produced greater biomass than each single biofilm, showing the existence of intergeneric synergistic interactions in biofilm formation. This result demonstrates that the parameters assessed by planktonic experiments did not allow prediction and generalization of the exact mechanism regulating mixed biofilm formation. Other cell-cell events, such as intergeneric coaggregation, may play a significant role in the formation and interspecies interactions in DW biofilms.

In conclusion, biofilms are recognized as focal points where bacteria and other organisms can interact (LeChevallier *et al.*, 1996; Heilmann *et al.*, 1997; Momba *et al.*, 2000; Berry *et al.*, 2006). This study provides evidences about some mechanisms by which different species interact in biofilms and should therefore help developing strategies for their elimination from the specific problem source. The development of multispecies

biofilms may help to determine optimal operational parameters and lead to knowledgeable decisions regarding the management of DW distribution networks that will guarantee microbiologically safe and thus high quality DW (LeChevallier *et al.*, 1996; Norton and LeChevallier, 2000; Berry *et al.*, 2006). The identification of the main bacteria forming more complex biofilms (*B. cepacia-M. mucogenicum, M. mucogenicum-Methylobacterium* sp. and *Methylobacterium* sp.-*A. calcoaceticus*) may provide new information necessary for improving water quality for the consumer.

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CHAPTER 8 CHEMICAL DISINFECTION TO CONTROL DRINKING WATER BIOFILMS

The knowledge of the role of microbial diversity of DW biofilms on disinfection might help to improve our understanding of their resistance mechanisms and allow the development of effective strategies to apply in DWDS. In this study six opportunistic bacteria (*Acinetobacter calcoaceticus, Burkholderia cepacia, Methylobacterium* sp., *Mycobacterium mucogenicum, Sphingomonas capsulata* and *Staphylococcus* sp.) isolated from a DWDS were used to form single and multispecies biofilms. The biofilms were exposed to SHC at different concentrations for 1 h and biofilm control was assessed in terms of mass removal and metabolic activity, cultivability and viability reduction. Biofilm recovery was also assessed 24 h after SHC treatment. The results demonstrate that total biofilm mass removal (single and multispecies biofilms) was not achieved for the SHC concentrations tested. Total biofilm inactivation was achieved only for *A. calcoaceticus* single-species biofilms and for those multispecies biofilms without *A. calcoaceticus*, when exposed to high SHC concentrations. From the single species biofilms, *Methylobacterium* sp. and *M. mucogenicum* had the highest resistance to SHC, while *Staphylocooccus* sp. and *A. calcoaceticus* formed the most susceptible biofilms. Multispecies biofilms with all the six bacteria had the highest resistance to SHC, while those without *A. calcoaceticus* were the most susceptible. In general, multispecies biofilms were more resistant to inactivation and removal than single species biofilms. The recovery results demonstrated that only biofilms without *A. calcoaceticus* were not able to recover their biomass from the SHC treatments. Also, those biofilms had a decreased ability to recover their viability. This study highlights the importance of *A. calcoaceticus* in the resistance and functional resilience of DW biofilms. Despite this bacterium being one of the most susceptible to SHC, its presence in multispecies biofilms increased their resistance to disinfection and their ability to recover from SHC exposure.

Part of the work presented in this chapter was published:

Simões LC, Simões M and Vieira MJ **(2010)** Influence of the diversity of bacterial isolates from drinking water on resistance of biofilms to disinfection. ***Applied and Environmental Microbiology*** **76:** 6673–6679.

8.1 Introduction

The control of DW quality in distribution systems is a major technological challenge to the water industry. DW networks can be regarded as biological reactors which host a wide variety of microorganisms (bacteria, protozoa and fungi), both in the bulk water and on the pipe surfaces (Amblard *et al.*, 1996; Block *et al.*, 1997; Berry *et al.*, 2006). In DWDS, *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Arthrobacter*/*Corynebacterium*, *Bacillus*, *Burkholderia*, *Citrobacter*, *Enterobacter*, *Flavobacterium*, *Klebsiella*, *Methylobacterium*, *Moraxella*, *Pseudomonas*, *Serratia*, *Staphylococcus*, *Mycobacterium*, *Sphingomonas* and *Xanthomonas* have been the predominant bacterial genera detected (Block *et al.*, 1997; Berry *et al.*, 2006). The Gram-negative are predominant over the Gram-positive bacteria, and *Pseudomonas* is the most abundant bacterial organism in supply systems, regardless the water source. Most of the biomass present in these DWDS is located at the pipe walls. Flemming *et al.* (2002) proposed that 95% of the bacteria were adhered to the surface of pipelines and only 5% was present in the bulk water. The presence and significance of biofilms in DWDS have been repeatedly reported (LeChevallier *et al.*, 1987; van der Wende and Characklis, 1990; Camper *et al.*, 1999; Momba *et al.*, 1999; Momba and Binda, 2002; Paris *et al.*, 2009).

Biofilms in DWDS may lead to a number of unwanted effects on the quality of the distributed water. Bacterial growth may affect the turbidity, taste, odour and colour of the water (Servais *et al.*, 1995; Emtiazi *et al.*, 2004), promote the corrosion of pipe materials (LeChevallier *et al.*, 1993; Beech and Sunner, 2004), induce a disinfectant demand and consequently promote disinfectant decay in distribution systems (Lu *et al.*, 1999; Chandy and Angles, 2001; Ndiongue *et al.*, 2005). Biofilm growth and detachment contribute to the increase in the number of cells in bulk water (van der Wende *et al.*, 1989; Chandy and Angles, 2001). Some of those microorganisms can be pathogens. Commonly encountered waterborne pathogens are *Burkholderia pseudomollei*, *Campylobacter* spp., *Escherichia coli*, *Helicobacter pylori*, *Legionella pneumophila*, *Mycobacterium avium*, *Pseudomonas aeruginosa*, *Salmonella* spp., *Shigella* spp., *Yersinia enterocolitica* and *Vibrio cholera* (WHO, 2008).Therefore, biofilm control is important for technical, aesthetic, regulatory and public health reasons.

The factors that may influence the development of biofilm in DWDS include the: concentration of biodegradable organic matter, disinfectant residual, water temperature, type

of pipe materials and their conservation state, hydraulic regime of system and the residence time of water (age of water in the system). According to some authors (Servais *et al.*, 1995; Ollos, 1998; Ndiongue *et al.*, 2005), biodegradable organic matter and the desinfectant residual are the key controlling factors of biofilm development in DWDS. A reduced level of organic matter decreases chlorine demand and increases the disinfectant stability, allowing the optimization of chlorine dosage and minimizes its depletion during distribution. Consequently, it improves the ability of chlorine to act against free or attached microorganisms (Codony *et al.*, 2005).

Chlorine disinfection is a key step in the biofilm control process. Residual concentrations must be kept below guidelines to lower the potential to form harmful disinfection by-products (Batterman *et al.*, 2000; Rand *et al.*, 2007; Rodríguez *et al.*, 2007). Chlorine, a strong oxidizing agent, is the most commonly used disinfectant due to its effectiveness, stability, easy of use and low cost. Furthermore, it can provide a residual disinfectant in water that prevents (or should prevent) microbial recovery from disinfection. Chlorine reacts with a variety of cellular components (proteins, lipids and nucleic acids) and affects several metabolic processes such as membrane permeability, ATPase activity, respiration and proton motive force of the cell (Ridgway and Olson, 1982; Shang and Blatchley III, 1999; Le Dantec *et al.*, 2002; Phe *et al.*, 2004, 2009). The cytoplasmic membrane has been proposed to be a possible key target involved in bacterial inactivation by chlorine, since alterations in its permeability after chlorination have frequently been described (Venkobachar *et al.*, 1997; Virto *et al.*, 2005). However, biofilm formation and their resistance to disinfection have been recognized as important factors that contribute to the survival and persistence of microbial contaminations in DW (Berry *et al.*, 2006). This inherent resistance to antimicrobials is mediated through many factors such as the metabolic changes induced by the bacterial attachment process; the direct interactions between the biofilm extracellular polymeric matrix constituents and antimicrobials, affecting diffusion and availability; the existence of nutrient microenvironments within the biofilm leading to areas of reduced or no growth (dormant cells); the existence of biofilm-specific phenotypes (strain variation) (Lewis, 2001; Simões *et al.*, 2009). Recent studies into the microbial ecology of DWDS have found that microbial resistance to disinfectants is also affected by microbial community diversity and interspecies relationships (Berry *et al.*, 2006; Simões *et al.*, 2007c). The dynamics of the microbial growth and multispecies biofilm formation in DW networks is very complex, as a large number of interacting processes are involved

(Regan *et al.*, 2003; Emtiazi *et al.*, 2004). Research into DW biofilm control will help to determine optimal disinfection parameters and lead to knowledgeable decisions regarding the management of DW distribution networks that will guarantee microbe-safe and high-quality DW.

The main purpose of this work was to understand the impact of microbial diversity of DW biofilms on their resistance to disinfection. The effects of SHC on the control of single and multispecies biofilms formed by DW-isolated bacteria, recognized as problematic opportunistic bacteria and with the potential to cause public health problems, were studied. Also, the biofilm ability to recover from SHC treatment was assessed.

8.2 Material and methods

8.2.1 Bacteria and bacterial cell growth

The bacteria used throughout this work were isolated from a model laboratory DWDS, as described previously in chapter 3 and by Simões *et al.* (2006). The isolates were identified by 16S rDNA gene sequencing according to the method described in chapter 4 and by Simões *et al.* (2007a). The assays were performed with six representative DW-isolated bacteria, *Acinetobacter calcoaceticus*, *Burkholderia cepacia*, *Methylobacterium sp.*, *Mycobacterium mucogenicum*, *Sphingomonas capsulata* and *Staphylococcus sp.*, respectively. Bacterial cells were grown overnight in batch cultures using 100 ml of R2A broth, at room temperature (23 ± 2 ºC) and under agitation (150 rpm). Afterwards, the bacteria were harvested by centrifugation (20 min at 13000 g, 4 ºC), washed three times in 0.1 M of PBS (KH_2PO_4; Na_2HPO_4, NaCl), and resuspended in a certain volume of R2A broth to obtain a cellular density of 10^8 cells ml^{-1}.

8.2.2 Chemical disinfectant

The chemical disinfectant used was SHC. A stock solution of SHC was prepared by diluting a commercially available solution (Sigma, Portugal) with sterile distilled water. Disinfectant solutions at various concentrations (0.1, 0.5, 1 and 10 mg l^{-1}) were prepared on the day of use and stored in the dark at 4 ºC.

8.2.3 Single and multispecies biofilm formation

Single and multispecies biofilms were developed according to the modified microtiter plate test proposed by Stepanović *et al.* (2000) using R2A broth as growth medium. Single-species biofilm formation was carried out with the six DW-isolated bacteria, and multispecies biofilms were developed at seven different bacterial combinations: one mixture of all six bacteria and six combinations with a mixture of five distinct bacteria through a strain exclusion process (biofilm formation in the absence of a specific strain, obtaining distinct species combinations) (Simões *et al.*, 2007b). For each condition, the wells of a sterile 96-well-flat-tissue culture plates (polystyrene, Orange Scientific, USA) were filled under aseptic conditions with 200 µl of a cell suspension (10^8 cells ml^{-1}). Multispecies biofilms were developed with equal initial cell densities of each isolate. Negative controls were obtained by incubating the wells with R2A broth without adding any bacterial cells. To promote biofilm formation, plates were incubated aerobically on an orbital shaker at 150 rpm and room temperature for 72 h. The growth medium was carefully discarded and freshly added every 24 h. All experiments were performed in triplicate with at least three repeats.

After the biofilm formation period, the content of each well was removed and the wells were washed three times with 250 µl of sterile distilled water to remove reversibly adherent bacteria. The remaining attached bacteria on the inner walls of the wells were submitted to the disinfection assay.

8.2.4 Biofilm disinfection assays

The biofilms, immediately after rinsing, were exposed to several independent SHC concentrations. At least 16 wells of 96-well microtiter plate were filled under aseptic conditions with 250 µl of each concentration of SHC. In addition to the treated wells, control (untreated) biofilm wells were also used for each biofilm condition. The SHC solutions remained in contact with the biofilms for 1 h but were removed and refreshed every 20 min during the 1 h treatment period. SHC solutions were refreshed due to the high density of cells in the biofilms and the low volumes applied for treatment (Shakeri *et al.*, 2007). In order to improve the contact of biofilm cells with SHC, the microtiter plates were incubated on a shaker at 150 rpm and at room temperature. After treatment, the disinfectant

solutions were removed by rinsing the wells twice with 250 µl of sodium thiosulfate solution (Merck, VWR, Portugal) at 0.5% (w v^{-1}) in sterile distilled water to quench the activity of the disinfectant and one time with 250 µl of sterile distilled water. Afterwards, the biofilms were analysed in terms of biomass, metabolic activity, cultivability and viability.

8.2.5 Biofilm recovery

The ability of biofilms to recover from disinfection was assessed 24 h after their exposure to the several independent SHC solutions. Microtiter plates with treated biofilms were filled under aseptic conditions with 200 µl of fresh R2A broth and were once more incubated aerobically on an orbital shaker, at 150 rpm and room temperature, for 24 h. After this period the content of each well was removed and the wells were washed three times with 250 µl of sterile distilled water to remove reversibly adherent bacteria. The remaining attached bacteria were assessed by the same techniques used to characterize the biofilms after disinfection.

8.2.6 Biomass quantification by CV

The bacterial biofilms in the 96-wells polystyrene microtiter plates were fixed with 250 µl of 98% methanol (Vaz Pereira, Portugal) per well for 15 min. Afterwards, the plates were emptied and left to dry. Then, the fixed bacteria were stained for 5 min with 200 µl of CV (Gram-colour-staining set for microscopy, Merck) per well. Excess stain was rinsed off by placing the plate under running tap water (Stepanović *et al.*, 2000). After the plates were air dried, the dye bound to the adherent cells was resolubilized with 200 µl of 33% (v v^{-1}) glacial acetic acid (Merck, Portugal) per well.

The OD of the obtained solutions were measured at 570 nm using a microtiter plate reader (BIO-TEK, Model Synergy HT) and biofilm mass were presented as OD_{570nm} values.

8.2.7 Metabolic activity assessment by XTT

The sodium 3,3'-[1[(phenylamino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) XTT colorimetric method was applied to determine the bacterial activity of the biofilms as

described previously by Stevens and Olsen (1993), with some modifications. The combined solution of XTT (Sigma) and PMS (Sigma) was added to each well, in order to obtain a final concentration of 50 μg ml^{-1} of XTT and 10 μg ml^{-1} of PMS. This solution was also added to each well of the 96-wells microtiter plates with bacterial biofilms. Then, the microtiter plates were incubated for 3 h, at 150 rpm, and room temperature, in the dark (Simões *et al*., 2007b). The OD of the formazan supernatant of each well was measured at 490 nm using a microtiter plate reader (BIO-TEK, Model Synergy HT). The biofilm specific respiratory activity was presented as $OD_{490\ nm/570\ nm}$ (biofilm metabolic activity/biofilm mass).

8.2.8 Biofilm cultivability assessment

The cultivability of bacterial biofilms was performed by the standard plate count method using R2A agar as culture medium. The biofilms in each well of microtiter plate were scraped with a sterile scalpel for 200 μl of sterile sodium phosphate buffer (pH 7). Before serial dilution, biofilm suspensions were vortexed for 2 min and then used to assess CFUs. CFUs were counted after 7-15 d incubation at room temperature to allow the enumeration of slow-growing bacteria adapted to oligotrophic conditions or oxidizing stressed bacteria, and the results were expressed as log CFU cm^{-2}.

8.2.9 Biofilm viability assessment by L/D staining

The viability of biofilms was assessed with L-7012 Live/Dead (L/D) *Bac*Light bacterial viability kit (Invitrogen/Molecular Probes, Leiden, Netherlands) using epifluorescence microscopy. This epifluorescence staining method allows the assessment of both viable and total counts of bacteria. The *Bac*Light kit is composed of two nucleic acid-binding stains: SYTO 9™ and propidium iodide (PI). SYTO 9™ penetrates all bacterial membranes and stains the cells green, while PI only penetrates cells with damaged membranes, and the combination of the two stains produces red fluorescing cells.

Biofilm suspensions, after vortexed for 2 min were diluted to an adequate concentration (in order to have 30–250 cells per microscopic field), being thereafter microfiltered through a Nucleopore (Whatman, Middlesex, UK) black polycarbonate membrane (pore size 0.22 μm), stained with 250 ml diluted solution of SYTO 9™ and 250

ml diluted solution of PI from the L/D kit, and left in the dark for 15 min. A microscope (Olympus BX51; Olympus, Japan), fitted with fluorescence illumination and a 40× fluorescence objective, was used to visualise the stained cells. The optical filter combination consisted of a 470–490 nm excitation filter, in combination with a LP516 nm emission filter with a barrier filter of 500 nm. Bacterial images were digitally recorded as micrographs using a microscope camera (Olympus, Japan). ScanPro5 (Sigma) was used to quantify the number of viable and nonviable cells. The mean number of viable and nonviable cells on each membrane was determined from counts of a minimum of 20 microscopic fields. The results were expressed as log cells cm^{-2}.

8.2.10 Calculations and statistical analysis

The SHC effectiveness (removal and inactivation) was assessed based on the absorbance values of the blank, the control experiment and the treated biofilm (Equation 1):

$$\text{Biofilm removal/inactivation (\%)} = \left[\frac{(C-B)-(T-B)}{(C-B)}\right]\times 100 \quad (1)$$

Where B indicates the average absorbance for the blank wells (without bacteria), C indicates the average absorbance for the control wells (untreated biofilms) and T indicates the average absorbance for the SHC-treated wells (Pitts *et al.*, 2003).

The ability of biofilms to recover from the treatment (percentage increase) was assessed from the absorbance values of the microtiter plate wells with the biofilms 24 h after disinfection, the biofilm immediately after disinfection and the blank (Equation 2):

$$\text{Biofilm regrowth (\%)} = \left[\frac{(RG-B)-(T-B)}{(T-B)}\right]\times 100 \quad (2)$$

RG indicates the average absorbance values of the mass of the biofilms 24 h after disinfection (regrowth experiments).

Biofilm control in terms of cultivability (CFU) and viability (L/D) was calculated by the following expression (Equation 3):

$$\text{Biofilm cultivability/viability reduction (\%)} = \left[\frac{\left(\text{CFUs or L/D}_{\text{control}} - \text{CFUs or L/D}_{\text{disinfection}}\right)}{\text{CFUs or L/D}_{\text{control}}}\right] \times 100 \quad (3)$$

While, the biofilm cultivability increase (recovery experiments) was assessed by the following equation (Equation 4):

$$\text{Biofilm cultivability increase (\%)} = \left[\frac{\left(\text{CFUs}_{\text{recovery}} - \text{CFUs}_{\text{disinfection}}\right)}{\text{CFUs}_{\text{disinfection}}}\right] \times 100 \quad (4)$$

The data were analysed using the statistical program SPSS version 14.0. The mean and standard deviation within samples were calculated for all cases. The data were analyzed by the nonparametric Wilcoxon test based on a confidence level of ≥ 95% ($P < 0.05$ was considered statistically significant).

8.3 Results

8.3.1 Single and multispecies biofilm removal

The tested bacteria formed single-species and multispecies biofilms with distinct susceptibilities to SHC (Figure 8.1).

The results indicate that biomass removal increased with increasing disinfectant concentration for all the biofilms, except for *A. calcoaceticus* single biofilms. These biofilms were significantly affected by disinfection, even for small SHC concentrations. *Methylobacterium* sp. formed the most resistant biofilms to SHC, for all the concentrations tested (Figure 8.1a). On the other hand, *A. calcoaceticus* formed the most susceptible biofilms to SHC up to a 1 mg l^{-1}, and *Staphylococcus* sp. biofilms were the most susceptible for the highest concentrations. The differences between the several single biofilms, in terms of mass removal, were less significant for the highest SHC concentrations ($P > 0.05$).

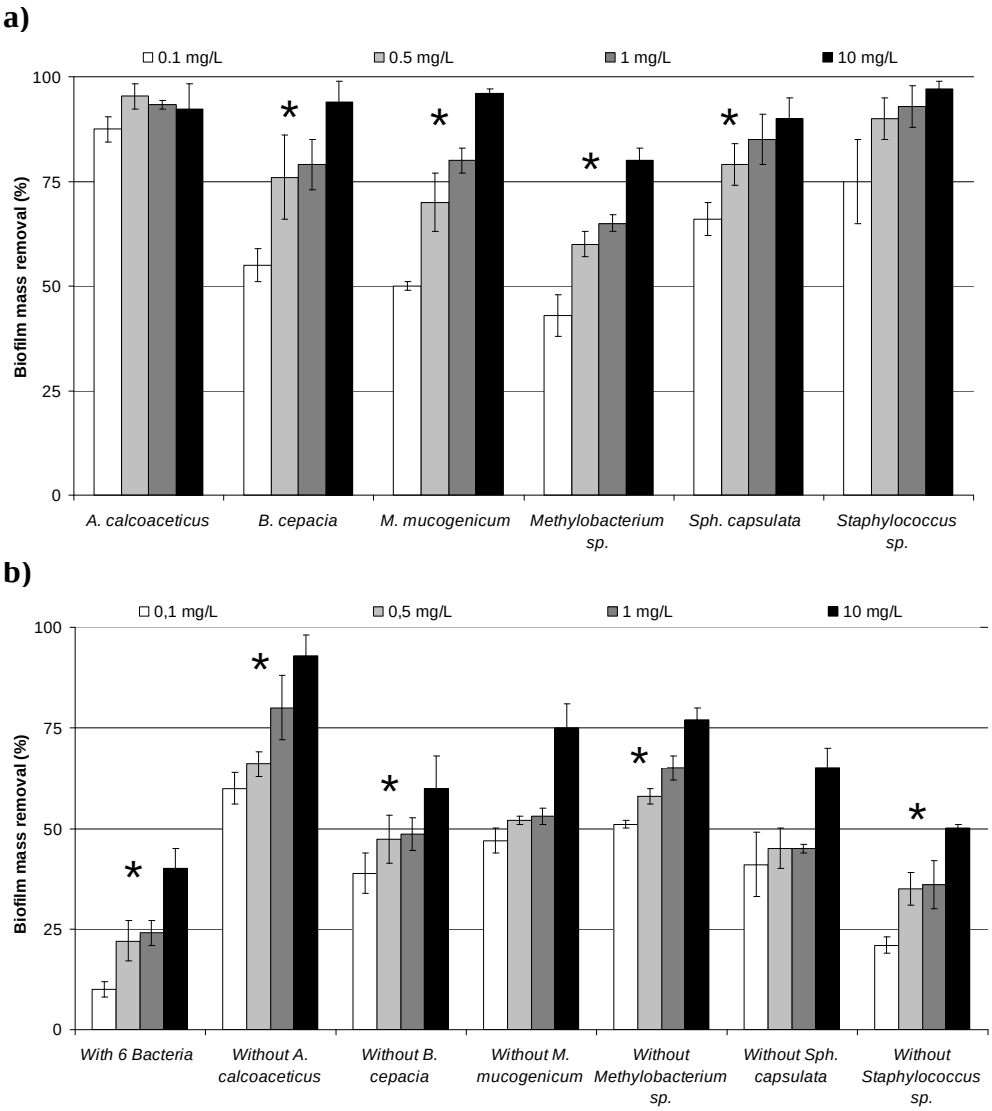

Figure 8.1 Percentage of biofilm mass removal for single species (a) or multispecies (b) biofilms after their exposure to several SHC concentrations. The means ± SDs for at least three replicates are illustrated. "*" indicates significant influence ($P < 0.05$) of SHC concentrations in biofilm removal.

For multispecies biofilms (Figure 8.1b), the order of susceptibility (from less to more susceptible) for all the SHC concentrations was the following: the biofilm with 6 bacteria, that without *Staphylococcus* sp., that without *B. cepacia* or *Sph. capsulata*, that without *M. mucogenicum*, that without *Methylobacterium* sp., and that without *A. calcoaceticus*. So, the bacterial combination with the six DW bacteria was the most resistant to SHC, and the least resistant was the bacterial combination without *A. calcoaceticus*, for all the concentrations tested.

Comparing single and multispecies biofilms (Figure 8.1a and b), almost all multispecies biofilms were more resistant to removal than the single biofilms ($P < 0.05$), except those multispecies biofilms without *M. mucogenicum* and without *Methylobacterium* sp. with 0.1 mg l^{-1} of SHC and multispecies biofilms without *A. calcoaceticus* for all the SHC concentrations tested ($P > 0.05$). These biofilms were more susceptible to chlorine than some of the single biofilms (*Methylobacterium* sp. [all concentrations], *M. mucogenicum* [0.1 mg l^{-1}], *B.cepacia* [0.1 and 1 mg l^{-1}], and *Sph. capsulata* and *A. calcoaceticus* [10 mg l^{-1}]).

8.3.2 Single and multispecies biofilm inactivation

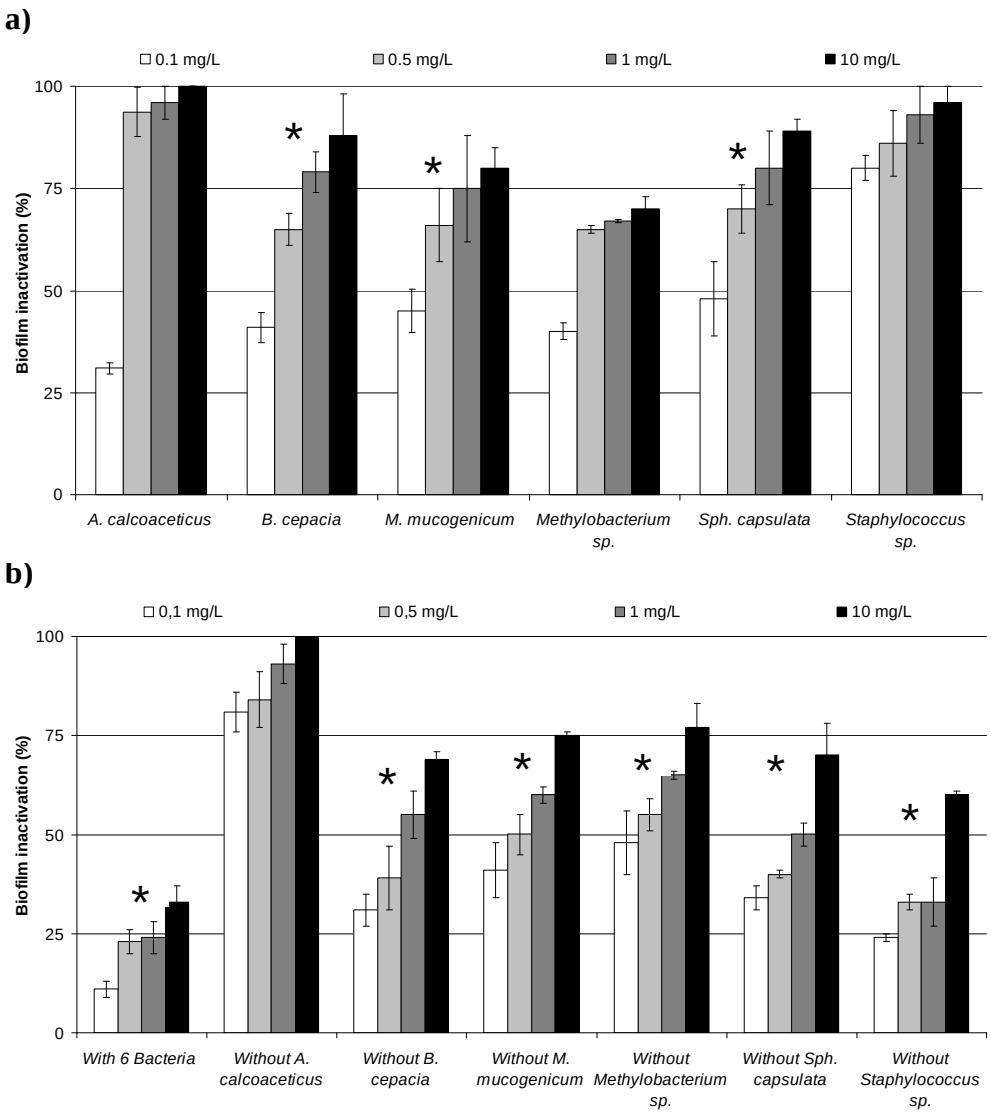

Figure 8.2 Percentage of biofilm inactivation for single species (a) or multispecies (b) biofilms after their exposure to several SHC concentrations. The means ± SDs for at least

three replicates are illustrated." *" indicates significant influence ($P < 0.05$) of SHC concentrations in biofilm inactivation.

Biofilm inactivation increased with SHC concentration for all the biofilms. *A. calcoaceticus* single biofilms presented the highest inactivation values for all the concentrations tested, with the exception of 0.1 mg l^{-1} (Figure 8.2a). For this concentration, *A. calcoaceticus* formed biofilms with the highest resistance to inactivation, while *Staphylococcus* sp. biofilms were the most susceptible. *Methylobacterium* sp. biofilms were the most resistant to disinfection at SHC concentrations higher than 0.1 mg l^{-1}. The sequence of resistance to inactivation for SHC concentrations of $\geq$ 1 mg l^{-1} was the following: *Methylobacterium* sp. was more resistant than *M. mucogenicum*, which was more resistant than *B. cepacia*, followed by *Sph. capsulata*, followed by *Staphylococcus* sp., followed by *A. calcoaceticus*. *A. calcoaceticus* biofilms reached total inactivation with SHC concentrations at 10 mg l^{-1}. Those biofilms were inactivated at significant extents (> 85%) even for small SHC concentrations (0.5 mg l^{-1}) (Figure 8.2a).

For multispecies biofilms (Figure 8.2b), the bacterial combination with the six bacteria was the most resistant to inactivation, followed by multispecies biofilms without *Staphylococcus* sp. The least resistant were the multispecies biofilms without *A. calcoaceticus*, followed by the biofilms without *Methylobacterium* sp, for all the SHC concentrations. When analysing Figure 8.2b it is perceptible that multispecies biofilms without *A. calcoaceticus* and the multispecies biofilms with all the six bacteria behave differently from the other multispecies biofilms ($P < 0.05$). The multispecies biofilms with all the six bacteria had the highest resistance to disinfection (even for high SHC concentrations only a 60% biofilm inactivation was obtained). Those without *A. calcoaceticus*, had a high susceptibility to SHC even for small concentrations (biofilm inactivation was always higher than 80%; total biofilm inactivation for SHC occurred at 10 mg l^{-1}).

In general, the multispecies biofilms were more resistance to inactivation than the single ones (Figure 8.2a and 8.2b). Multispecies biofilms without *A. calcoaceticus* were the most relevant exception. Those biofilms were more susceptible to disinfection at some SHC (0.1 mg l^{-1}) concentrations, than the single species biofilms ($P < 0.05$).

8.3.3 Single and multispecies biofilm cultivability and viability reduction

The single and multispecies biofilms were also characterized in terms of cultivable and viable cells counts by means of the standard plate count method and by the L/D staining method, respectively (Table 8.1).

Table 8.1 Initial (before disinfection) counts of single and multispecies cultivable and viable biofilm cells[a]

Biofilm description	Cultivable cell count	Viable cell count
Single biofilms		
A. calcoaceticus	5.08 ± 0.43	6.33 ± 0.05
B. cepacia	5.24 ± 0.33	6.41 ± 0.44
M. mucogenicum	4.37 ± 0.70	6.06 ± 0.66
Methylobacterium sp.	6.58 ± 0.55	7.69 ± 0.14
Sph. capsulata	5.70 ± 0.16	6.88 ± 0.32
Staphylococcus sp.	6.00 ± 0.38	7.21 ± 0.46
Multispecies biofilms		
With 6 bacteria	6.87 ± 0.23	7.91 ± 0.31
Without *A. calcoaceticus*	6.88 ± 0.11	7.97 ± 0.40
Without *B. cepacia*	7.03 ± 0.28	8.11 ± 0.26
Without *M. mucogenicum*	7.15 ± 0.36	8.51 ± 0.08
Without *Methylobacterium* sp.	6.70 ± 0.45	7.89 ± 0.68
Without *Sph. capsulata*	6.70 ± 0.16	7.78 ± 0.41
Without *Staphylococcus* sp.	7.23 ± 0.41	8.31 ± 0.55

[a]Values are expressed as log CFU cm^{-2} or log viable cells cm^{-2} ± SD.

The number of viable cells was higher than the number of cultivable cells for all single and multispecies biofilms (magnitude of difference of 1 to 2 log of cells cm^{-2}). The multispecies biofilms always displayed higher numbers of cultivable and viable cells than the single species. Also, L/D results demonstrate that before disinfection, almost all the bacteria in the several single and multispecies biofilms were in a viable state (99.9 ± 0.003%).

Biofilm cultivability and viability after disinfection provided results comparable with those obtained by XTT staining for all the biofilms ($P > 0.05$). It was also verified that biofilm cultivability and viability decreased with the increasing of SHC concentration.

Comparing the values obtained for metabolic inactivation (Figure 8.2), cultivability reduction (Figure 8.3), and viability reduction (Figure 8.4), the cultivability results provided the most promising biofilm control results for all the scenarios tested.

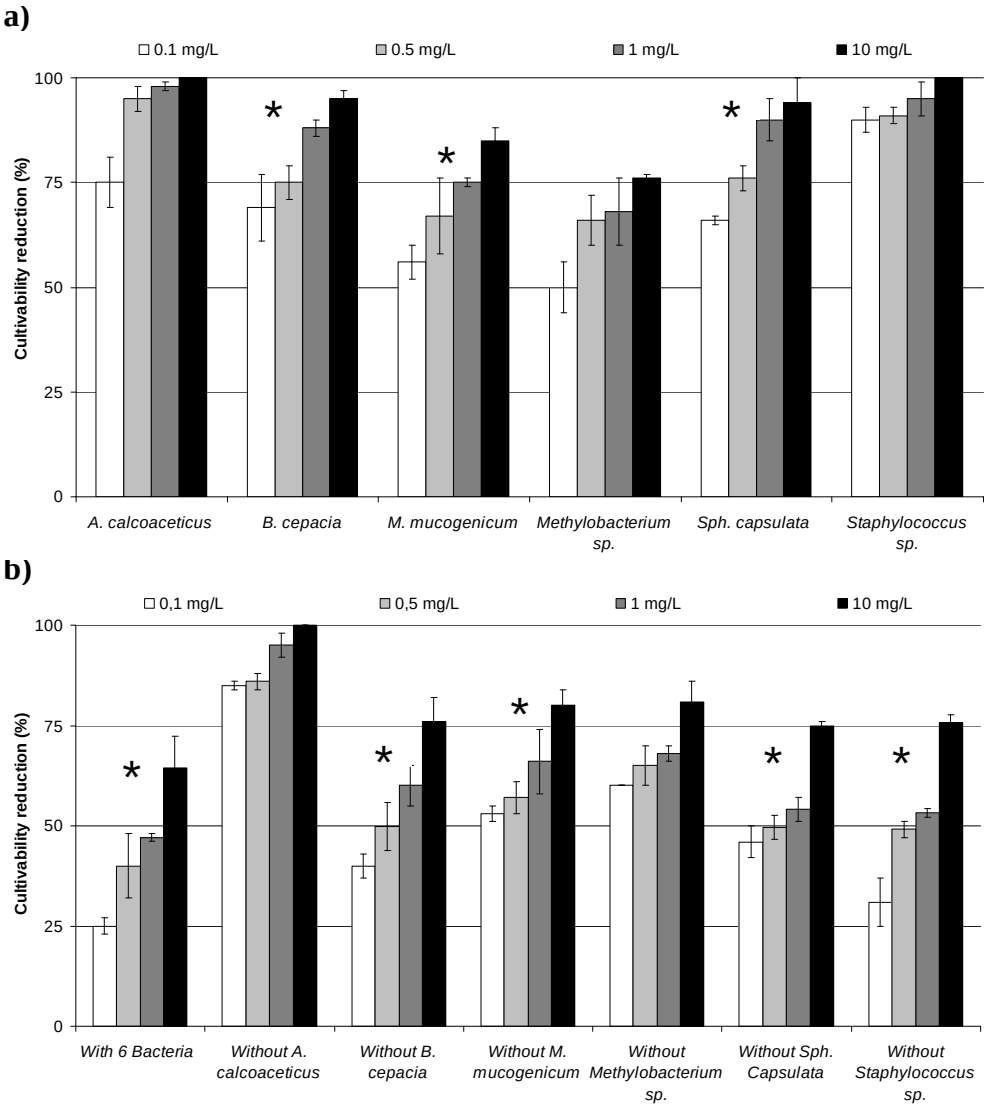

Figure 8.3 Percentage of cultivability for single species (a) or multispecies (b) biofilms after their exposure to several SHC concentrations. The means ± SDs for at least three replicates are illustrated." *" indicates significant influence ($P < 0.05$) of SHC concentrations in biofilm cultivability reduction.

The sequence of single biofilms concerning the cultivability reduction (Figure 8.3a) was similar to those obtained with XTT staining. *Methylobacterium* sp. biofilms were the most resistant to disinfection and *A. calcoaceticus* and *Staphylococcus* sp. biofilms were the most susceptible. The cultivability of *A. calcoaceticus* and *Staphylococcus* sp. biofilm cells decreased significantly (> 90%) even for the small SHC concentration (0.5 mg l^{-1}). Also,

were only those that achieved total loss of the cultivability (100%) (10 mg l^{-1}), the remaining biofilms did not achieve total loss of cultivability to SHC concentrations tested.

Regarding multispecies cultivability reduction (Figure 8.3b) the biofilms more resistant and more susceptible to disinfection were those already referred in the metabolic inactivation experiments, for all the concentrations tested. Comparing single and multispecies biofilm cultivability reduction (Figure 8.3a and b) multispecies biofilms were more resistant to disinfection than the single ones. Multispecies biofilms without *A. calcoaceticus* was the most relevant exception.

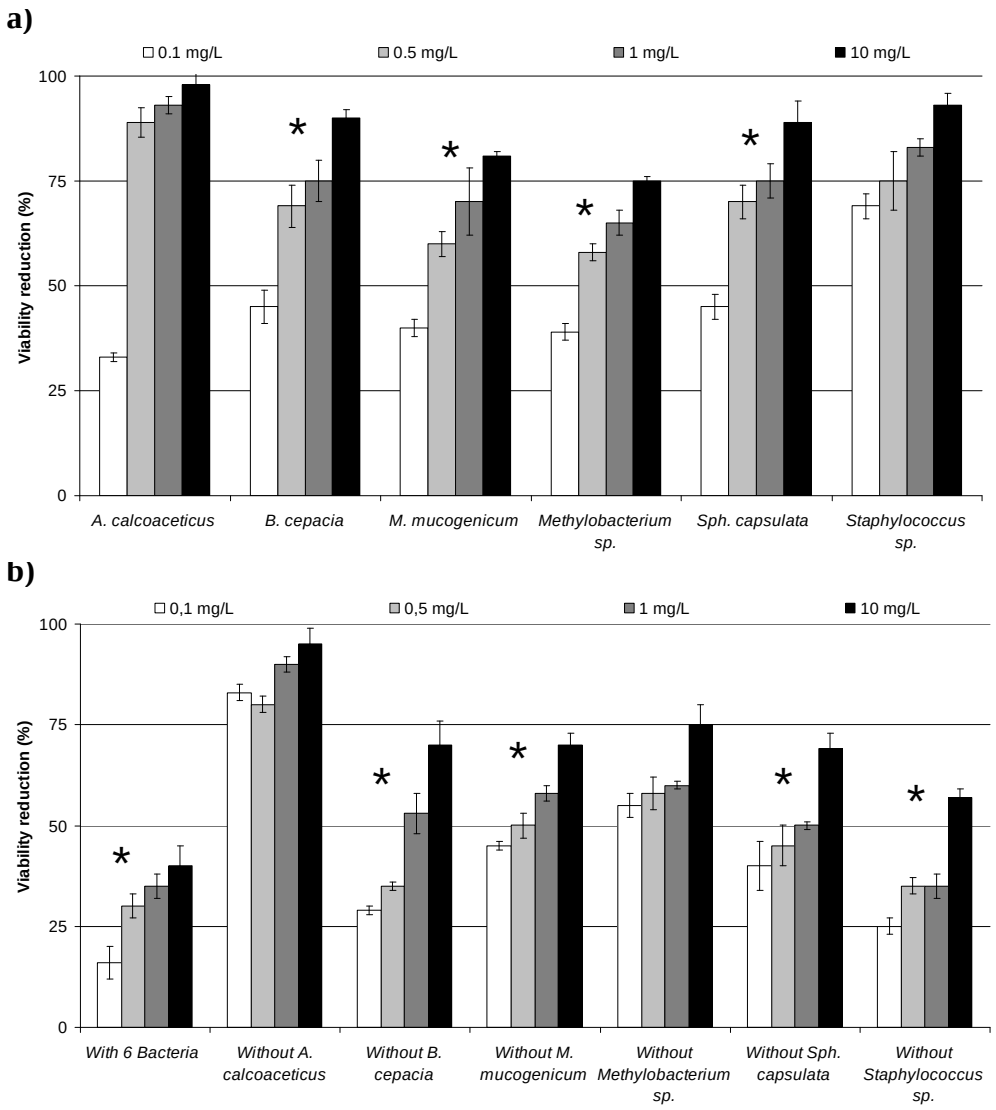

Figure 8.4 Percentage of viability for single species (a) or multispecies (b) biofilms after their exposure to several SHC concentrations. The means ± SDs for at least three replicates are illustrated." *" indicates significant influence ($P < 0.05$) of SHC concentrations in reduction of biofilm viability.

In terms of viability reduction and for SHC higher than 0.1 mg l^{-1}, *Methylobacterium* sp. biofilms were the most resistant and *A. calcoaceticus* formed the least resistant single biofilms (Figure 8.4a). Concerning the multispecies biofilms (Figure 8.4b), those with all the 6 bacteria were the most resistant, while multispecies biofilms without *A. calcoaceticus* were the least resistant, for all the tested SHC concentrations. The analysis of biofilm viability reduction corroborates the data obtained by XTT staining and CFU counts ($P > 0.05$). However, the viable cell counts provide the worst case scenario in terms of biofilm control analysis (higher number of viable cells for all the SHC concentrations tested).

8.3.4 Single and multispecies biofilm recovery from disinfection

In order to assess the ability of biofilms to recover from disinfection, the single and multispecies biofilms were exposed to fresh medium and allowed to grow for 24 h after treatment. After that, biofilms were characterized in terms of mass, metabolic activity, cultivability and viability. The data of biofilm recovery in terms of activity, culturability and viability provided comparable results ($P > 0.05$). Consequently, only cultivability results are described, providing an underestimated analysis of biofilm recovery comparatively to the results of metabolic activity and viability.

Figure 8.5a and 8.5b demonstrate the ability of some single and multispecies biofilms to recover in terms of biofilm mass. *A. calcoaceticus* and *Staphylococcus sp.* single biofilms had the ability to recover from the several SHC treatments (Figure 8.5a). The other single biofilms had no ability to recover from disinfection. Concerning the multispecies biofilms (Figure 8.5b), those without *A. calcoaceticus* were the only multispecies biofilms that had no ability to recover from disinfection for all the SHC concentrations. Conversely, multispecies biofilms without *Staphylococcus* sp. were able to recover from all the treatments and were the only that had a gradual increase of biofilm mass with SHC concentration ($P > 0.05$). These biofilms were the multispecies biofilms with the highest ability to recover from disinfection. The other multispecies biofilms had at least one SHC concentrations (0.1 mg l^{-1}) allowing biofilm mass recovery.

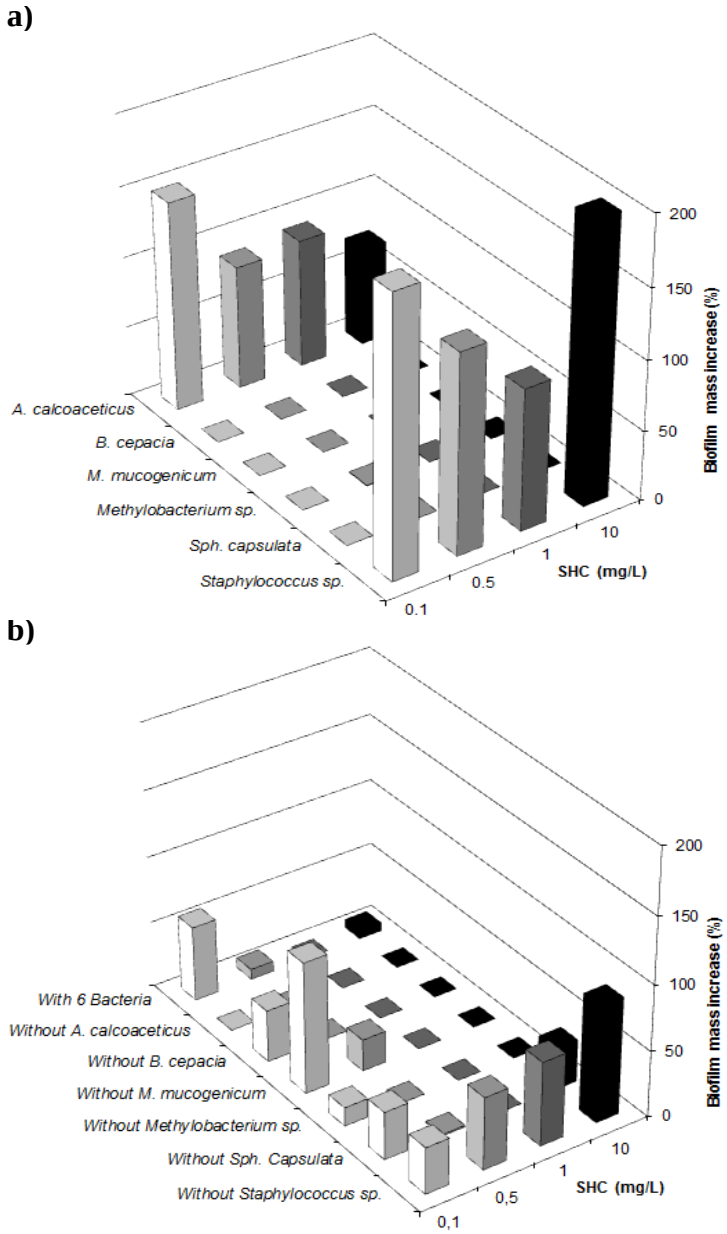

Figure 8.5 Percentage of biofilm mass increase for single (a) and multispecies (b) biofilms, 24 h after treatment with several SHC concentrations. The means ± SDs for at least three replicates are illustrated.

Biofilm mass and cultivability recovery were not correlated for all the biofilms tested ($P < 0.05$). All the single biofilms, except *Staphylococcus* sp. biofilms recovered significantly their cultivable population one day after SHC treatment ($P < 0.05$). *Staphylococcus* sp. biofilms previously exposed to SHC at 0.1 and 0.5 mg l^{-1} had no ability to recover (0%). *Sph. capsulta* and *A. calcoaceticus* were the biofilms with the highest ability to recover the number of cultivable cells after treatment. On the other hand, *Methylobacterium* sp. formed biofilms with the smallest ability to recover their cultivability.

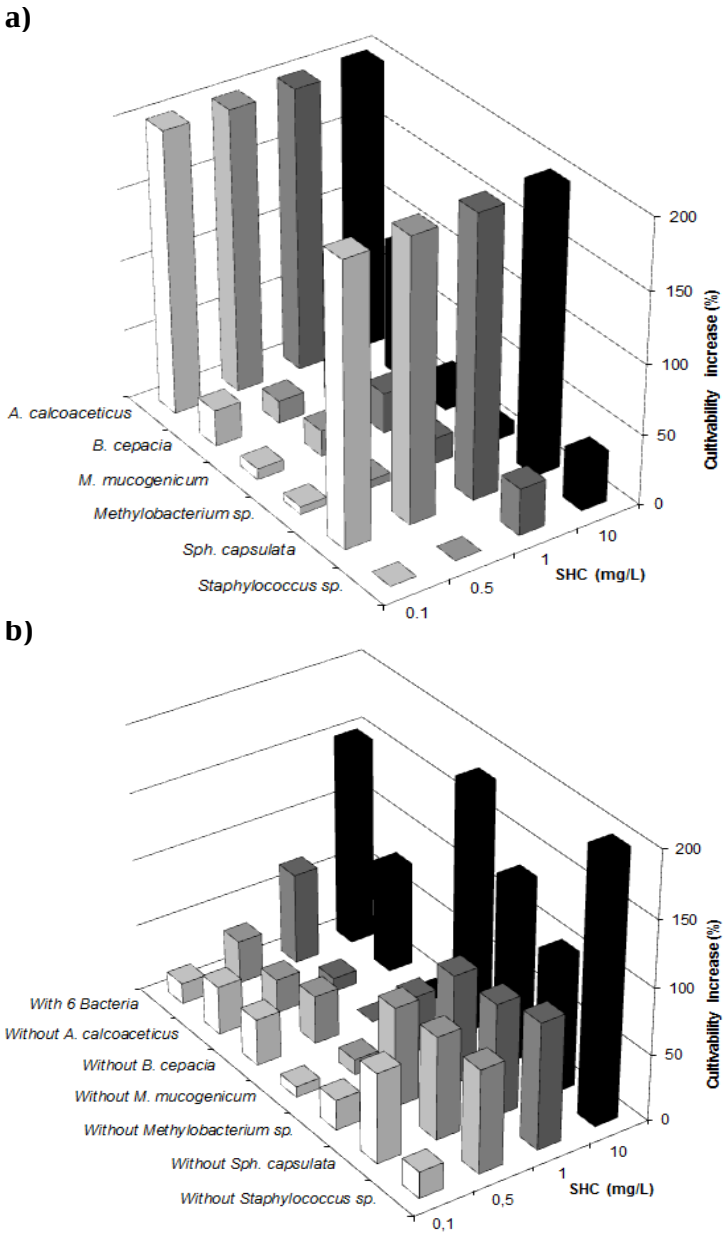

Figure 8.6 Percentage of cultivability increase for single (a) and multispecies (b) biofilms, 24 h after treatment with several SHC concentrations. The means ± SDs for at least three replicates are illustrated.

All the multispecies biofilms had the ability to recover the number of cultivable cells from SHC exposure, except those without *B. cepacia* for 1 mg l^{-1} of SHC (Figure 8.6b). SHC at 0.1 mg l^{-1} allowed the maximum cultivability recovery of multispecies biofilms without *Sph. capsulata* and without *A. calcoaceticus*. For SHC concentrations higher than 0.1 mg l^{-1}, multispecies biofilms without *Staphylococcus* sp. were those with the highest recovery potential, followed by the multispecies biofilms without *Sph. capsulata* (0.5 and 1 mg l^{-1}) and without *M. mucogenicum* (10 mg l^{-1}). The multispecies biofilms with the lowest

ability to restore after disinfection were those without *M. mucogenicum* (0.1 and 0.5 mg l^{-1}) and without *B. cepacia* (1 and 10 mg l^{-1}). The number of cultivable cells in multispecies biofilms without *A. calcoaceticus* decreased 1 day after treatment and with the increase of SHC concentration, comparatively to the other multispecies biofilms. Multispecies biofilms without *A. calcoaceticus* had a significant ability to recover from SHC exposure at low concentrations (0.1 mg l^{-1}) and the lowest ability to recover from the application of the highest concentration (10 mg l^{-1}).

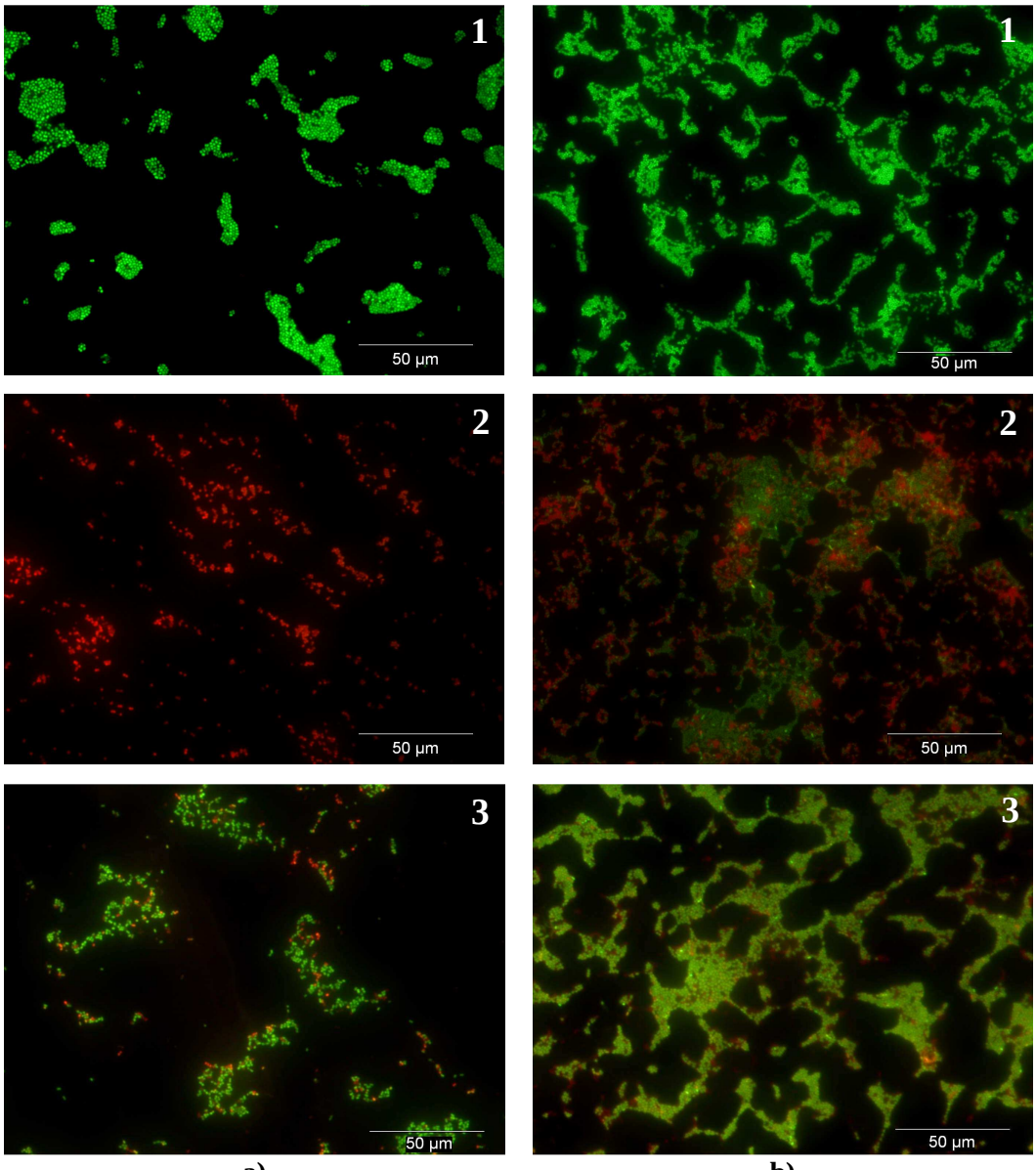

Figure 8.7 Epifluorescence photomicrographs of *A. calcoaceticus* biofilm cells (a) and multispecies biofilms with all the six bacteria (b), before treatment with 1 mg l^{-1} SHC (1); immediately after (2) and 1 day later (3). Magnification, ×400; bar = 50 µm. Viable cells are green and non-viable cells are red.

The phenomenon of biofilm recovery was also evident when single and multispecies biofilms were stained with a viability stain and observed by epifluorescence microscopy. Figure 8.7 depicts the epifluorescence photomicrographs concerning one representative example of the effects of SHC (1 mg l^{-1}) on the viability of *A. calcoaceticus* single biofilms (Figure 8.7a) and of multispecies (Figure 8.7b) biofilms composed by the six bacteria. The proportion of viable cells (green) decreased significantly becoming non-viable and/or injured (red). However, the spatial amount of non-viable cells or injured cells clearly decreased 24 h after disinfection, increasing the proportion of viable cells. Comparing the single and multispecies biofilms, it is clearly the lower effects of SHC on multispecies biofilms immediately after the treatment. The multispecies biofilms with all the six bacteria had a significant amount of green cells after SHC exposure, while the majority of *A. calcoaceticus* are red. However, both biofilms had a significant ability to recover 24 h after SHC exposure.

8.4 Discussion

Understanding how biofilms respond to external stress conditions is essential for the development of effective control strategies. The present study has implications for understanding the role of microbial diversity on biofilms formed by DW-isolated bacteria in their susceptibility to SHC. DWDS are known to harbour biofilms, even though these environments are oligotrophic and often contain a disinfectant. Control of these biofilms is important for aesthetic and regulatory reasons. DW distribution networks may be viewed as a large reactor where a number of chemical and microbiological processes are taking place. Control of microbial growth in DWDS, often achieved through the addition of disinfectants, is essential to limit the spread of waterborne pathogens. Some authors referred the presence of the disinfectant as the most important factor which limits the increase of biofilm formation and the number of cells in the bulk water in DWDS (Codony *et al.*, 2002; Zhang and DiGiano, 2002; Zhou *et al.*, 2009). However, microorganisms can resist disinfection through protection within biofilms. Therefore, the maintenance of chlorine residual at levels normally recommended cannot be relied upon to totally prevent the occurrence of bacteria.

The SHC concentrations used were those usually present in DWDS, with the exception of the highest concentration (10 mg l^{-1}). This was used to promote significant

biofilm removal and inactivation results, taking into account the high cell densities of the biofilms formed on the microtiter plates (increasing the ratio of SHC per amount of biofilm). According to the WHO (2008), 2 to 3 mg l^{-1} of chlorine should be added to water in order to provide a satisfactory disinfection and a residual concentration along a DWDS. However, the maximum amount of chlorine one can use is 5 mg l^{-1}. For a more effective disinfection the residual amount of free chlorine should exceed 0.5 mg l^{-1} after at least 30 minutes of contact time at pH value of 8 or less (WHO, 2008).

This study was developed using polystyrene microtiter plates, which are the most frequently used bioreactor system for studying biofilm formation and disinfection, providing reliable comparative data (Pitts *et al.*, 2003; Shakeri *et al.*, 2007; Dror-Ehre *et al.*, 2010). Microtiter plates can be used as a rapid and simple method to screen the differences in efficiency of chlorine to remove and kill different biofilms and to display their ability to recover from chlorine treatment. Polystyrene has physico-chemical surface properties similar to those of other materials used in DWDS such as stainless steel and polyvinylchloride (Simões *et al.*, 2007a).

In order to assess the efficacy of SHC disinfection on DW single and multispecies biofilms and their ability to recover from the treatment, four different methods were used to characterize the biofilms. These included two relatively rapid assays: staining with CV, as indicator of total attached biomass (Djordjevic *et al.*, 2002; Pitts *et al.*, 2003; Stepanović *et al.*, 2004) and respiratory activity assessment by staining with XTT, as indicator of the metabolic activity of the attached biomass (Berit *et al.*, 2002; Pettit *et al.*, 2009). Also, the traditional plate count technique was used to detect the number of cultivable biofilm bacteria and the epifluorescence staining method using L/D was used to assess the number of viable bacteria (Boulos *et al.*, 1999; Gião *et al.*, 2009). The differences provided by viability and cultivability methods allowed the assessment of the number of non-cultivable but metabolically active cells, classically called “viable but non-cultivable” (VBNC), which exist in response to chlorine stress (Leriche and Carpinter, 1995). These cells can be either temporarily non-cultivable, cultivable under other culture conditions, or simply dead cells (McDougald *et al.*, 1998). Lindsay *et al.* (2002) highlighted the importance of taking into account the injured cell population during disinfection as such populations may recover and recolonize the surfaces. Plate count techniques are known to be inefficient in the detection of disinfectant-injured bacteria and can overestimate disinfection (McFeters, 1990; McFeters *et al.*, 1995; Simões *et al.*, 2005). This is apparently related to the existence of

VBNC cells. According to Thomas *et al.* (2002), these cells constitute the most numerically significant and persistent sub-population within the aquatic systems.

Although the results obtained by the several methods correlated with each other, there are some differences between them. CV staining was the only method that provided the total biomass amount. The other methods provided information on the physiological state of the biofilm cells. The percentage of biofilm mass removal was smaller than the inactivation in most situations. Total biofilm mass removal (single and multispecies biofilms) was not achieved for the SHC concentrations tested. These differences in biofilm removal and inactivation was an expected result as the cells that are removed cannot contribute to activity whereas cells that have lost activity can still contribute to the total amount of biomass. Total biofilm inactivation was only achieved at 10 mg l^{-1} of SHC for *A. calcoaceticus* single species biofilms and for multispecies biofilms without *A. calcoaceticus*. Plate counts data yielded the most optimistic estimate of biofilm disinfection and overestimate SHC efficacy comparatively to the other methods. This indicates that many bacterial cells determined to be incapable of producing a colony on agar plate still exhibited some respiratory activity. This is in accordance with previous works (Yu *et al.*, 1993; Stewart *et al.*, 1994). However, even if the cell counts based on culture methods underestimate the number of bacteria, some authors argue that they could be used as a general indicator that demonstrates the efficiency of disinfection in DWDS (Ashbolt *et al.*, 2001; Codony *et al.*, 2005). The active biofilm left behind may constitute a source of additional problems such as recovery and regrowth, development of resistant biofilms or harbour for other microorganisms, including problematic pathogens (Walker *et al.*, 2003; Camper, 2004; Møretrø and Langsrud, 2004; Lapidot *et al.*, 2006). The survival of some bacterial cells following SHC exposure, verified by the recovery tests, allowed biofilm regeneration and thus permitted recovery in terms of respiratory activity, cultivability and viability. According to Stewart (2003), a reduced and reversible antimicrobial susceptibility could lead to populations of resistant bacteria, which may be recalcitrant to further disinfection processes.

Multispecies biofilms were more resistant to inactivation and removal by SHC than the single biofilms. The increasing resistance of multispecies biofilms can be partly explained by the higher cell densities relative to those of single biofilms. The cell densities of the multispecies biofilms were higher than those of the single ones for all the biofilm tested. Other potential reasons for the increased resistance of biofilm cells to antimicrobials

include the difficulty to penetrate the matrix surrounding the biofilms by the disinfectant, the altered microenvironment, which in turn contributes to slow microbial growth, the acquisition of resistance phenotypes, and the existence of persistent cells (Samrakandi *et al.*, 1997; Lewis, 2001; Stewart *et al.*, 2004; Shakeri *et al.*, 2007). Also, the interactions in multispecies biofilms may influence each other not only with respect to attachment capabilities but also in susceptibility or resistance to a disinfectant (Lindsay *et al.*, 2002; Molin *et al.*, 2004; Burmølle *et al.*, 2006; Chorianopoulos *et al.*, 2008). According to Shakeri *et al.* (2007), the higher resistance of a multispecies biofilm than of single species biofilms depends on the variation in the species incorporated and the role of each species. This may be due to the resistance of only one or two key strains. Leriche and Carpentier (1995) demonstrated that *P. fluorescens* and *Salmonella Typhimurium* in biofilm enhanced each other's survival following chlorine treatment. The co-culturing of the two bacteria in biofilm enhanced resistance of the individual strains to disinfection. *Staphylococcus sciuri* was also found to protect *Kocuria* species microcolonies against a chlorinated alkaline solution (Leriche *et al.*, 2003). Other apparent protective effects caused by bacterial association have been mentioned (Whiteley *et al.*, 2001; Lindsay *et al.*, 2002; Simões *et al.*, 2009). The synergistic species association found in this study, in addition to other well-described biofilm specific antimicrobial resistance mechanisms (Mah and O'Toole, 2001; Cloete, 2003; Davies, 2003; Klapper *et al.*, 2007), could at least partly explain the survival of complex multispecies biofilms in adverse environments.

The comparison of the SHC susceptibilities of multispecies biofilms shows that biofilms composed by the six different species had the highest resistant to removal and inactivation. In fact, the results demonstrate that biofilm species association/diversity promotes community stability and functional resilience even after SHC treatment. Biofilms in the absence of *Staphylococcus* sp. had a significant resistance to SHC exposure. On the other hand, *Staphylococcus* sp. single biofilms were highly susceptible to SHC. This result is arguably related to the higher susceptibility of Gram-positive to multi-target antimicrobials comparatively to that of Gram-negative bacteria (Virto *et al.*, 2005). Whereas the envelopes of Gram-positive bacteria consist of the cytoplasmic membrane surrounded by a thick peptidoglycan wall, the envelopes of Gram-negative bacteria possess an external layer, the outer membrane, which provides an extra barrier against antimicrobials. The most susceptible multispecies biofilms were those lacking *A. calcoaceticus*, *Methylobacterium* sp. and *M. mucogenicum*. The absence of these bacteria in the multispecies biofilm increased

the susceptibility to SHC. *A. calcoaceticus* biofilms were significantly affected by chlorine even at small SHC concentrations. This bacterium was one of the most susceptible. On the other hand, multispecies biofilms that lacked *A. calcoaceticus* led the most SHC susceptible biofilms and showed a decreased ability to recover from disinfection. This can be explained by the role of *A. calcoaceticus* as a bridging bacterium in this microbial community. In a previous study, it was demonstrated that this bacterium has the ability to coaggregate with almost all other bacteria (except *Methylobacterium* sp.), and its presence in a multispecies community represented a colonization advantage (Simões *et al.*, 2008). This bacterium may facilitate the association of the other species that do not coaggregate directly with each other, increasing the opportunity for metabolic cooperation. Bacterial coaggregation in well-established microbial biofilm communities seems to be one potential synergistic interaction that not only promotes their growth but also improves their resistance to SHC disinfection (Özok *et al.*, 2007). *Methylobacterium* sp. and *M. mucogenicum* single biofilms were the most resistant to SHC. The increased resistance demonstrated by these bacteria can arguably be related to their ability to form biofilms with the highest cell densities. Also, *Methylobacterium* sp. had the lowest doubling time (chapter 7). According to Taylor *et al.* (2000), the more slowly growing strains are more resistant to chlorine than the rapidly growing strains. Hiraishi *et al.* (1995) verified that *Methylobacterium* isolates derived from chlorinated water supplies exhibited higher resistance to chlorine than other isolates from different environments. *Mycobacteria* are among the least susceptible cell types, due to the innate presence of a waxy cell envelope (Le Dantec *et al.*, 2002).

A. calcoaceticus formed biofilms susceptible to SHC. However, the biofilms (single and multispecies) formed by this bacterium had the highest potential to recover from SHC exposure. The presence of *A. calcoaceticus* in the biofilm community increased the ability of multispecies biofilms to recover from disinfection. Multispecies biofilms without *A. calcoaceticus* was the only biofilm that had no ability to recover in terms of mass and had the lowest ability to recover their cultivability. This fact reinforces the role of *A. calcoaceticus* on the resilience of DW biofilms and their influence in recovery from disinfection. *Methylobacterium* sp. and *M. mucogenicum* (single and multispecies) biofilms had the highest resistance to SHC. However, their single species biofilms had low ability to regrowth and their multispecies biofilms had variable recovery potentials (depending of which species were present). The synergistic interspecies interactions found in multispecies

biofilms influence each other with respect to disinfectant susceptibility and also on their ability to recover from disinfection.

In conclusion, knowledge of biofilm microbial diversity and behaviour can contribute to the design of effective control strategies (able to control the key microorganisms in the resistance and resilience of a biofilm, such as *A. calcoaceticus*) that will guarantee safe and high-quality DW. Often, the mechanisms responsible for the survival of bacteria in DW supplies are unknown or poorly understood. Some authors already have proposed that this increased resistance to disinfection may result from the microbial diversity and microbial interactions in well-established consortiums adhered on the walls of water pipes (Berry *et al.*, 2006; Simões *et al.*, 2007c). To our knowledge, this is the first report providing experimental evidence on the role of the microbial diversity of DW-isolated bacteria biofilms on their resistance to SHC disinfection.

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CHAPTER 9 BACTERIAL METABOLITES TO CONTROL DRINKING WATER BIOFILMS

The knowledge of the mechanisms by which diverse species survive and interact in DW biofilm communities may allow to identify new biofilm control strategies. The purpose of this study is to understand the effects of metabolite molecules produced by the bacteria on biofilm formation. In this study six opportunistic bacteria (*Acinetobacter calcoaceticus*, *Burkholderia cepacia*, *Methylobacterium* sp., *Mycobacterium mucogenicum*, *Sphingomonas capsulata* and *Staphylococcus* sp.) isolated from a DWDS were used to form single and multispecies biofilms in the presence and absence of cell-free supernatants produced by the partner bacteria. The biofilms were assessed in terms of mass and metabolic activity. Additionally, several bacterial physiological aspects regulating interspecies interactions (planktonic and sessile growth rates, cell-free supernatant antimicrobial activity, and production of QSI, QS molecules and iron chelators) were characterized with the intent to identify bacterial species with biocontrol potential in DWDS. In terms of physiological characteristics, *B. cepacia* showed the highest planktonic growth rate and *A. calcoaceticus* the smallest. *Methylobacterium* sp. biofilms had the highest growth rate and *M. mucogenicum* biofilms the lowest. *B. cepacia*, *Methylobacterium* sp., *Sph. capsulata*, and *Staphylococcus* sp. produced QSI molecules and only *B. cepacia* was able to produce extracellular iron-chelating molecules. *A. calcoaceticus*, *B. cepacia*, *Methylobacterium* sp. and *M. mucogenicum* biofilms were strongly inhibited by the cell-free supernatants from the other bacteria. *M. mucogenicum* and *Sph. capsulata* cell-free supernatants demonstrated a high potential to inhibit the growth of counterpart biofilms. For multispecies biofilms without cell-free supernatants only those in the absence of *A. calcoaceticus* were strongly inhibited. Multispecies biofilms were highly susceptible in the absence of *A. calcoaceticus*. Only cell-free supernatants produced by *B. cepacia* and *A. calcoaceticus* had no inhibitory effects (caused potentiation) on multispecies biofilm formation. The overall report demonstrates the role of key bacteria, particularly *A. calcoaceticus*, on the multispecies biofilm community and the ability of bacteria to produce extracelular metabolite molecules with significant impact in biofilm formation and development.

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9.1 Introduction

Bacterial biofilms are complex communities of microorganisms embedded in a self-produced matrix and adhering to inert or living surfaces (Costerton *et al.*, 1999). Biofilms have been observed on a variety of surfaces and in diverse niches, and are considered to be the prevailing microbial lifestyle in most environments (van Houdt *et al.*, 2004). This sessile mode of life allows bacteria to enjoy a number of advantages over their planktonic counterparts, particularly the increased resistance to antimicrobial agents (Gilbert *et al.*, 2002; Chorianopoulos *et al.*, 2010; Ferreira *et al.*, 2010).

DW pipes inner-surfaces are invariably colonized by biofilms, regardless the presence of a disinfectant residual. Biofilms on DWDS may lead to a number of unwanted effects on the quality of the distributed water. In addition to the possibility of causing corrosion, turbidity, taste and odour problems, biofilms control the microbiological contents of the distributed water and are a potential source of pathogens (Percival and Walker, 1999; Szewzyk *et al.*, 2000; Manuel *et al.*, 2009).

DW networks can be regarded as biological reactors which host a wide variety of microorganisms (bacteria, protozoa, fungi) (Amblard *et al.*, 1996; Block *et al.*, 1997). Microbial diversity leads to a variety of complex relationships involving interspecies and intraspecies interactions (Berry *et al.*, 2006; Elenter *et al.*, 2007; Hansen *et al.*, 2007; Simões *et al.*, 2007b). Interactions among bacterial species may have a profound influence on the initial stages of biofilm formation and development. The surface colonization by bacteria can enhance the attachment of others to the same surface. Some bacteria had an important role in development of biofilm formation by DW-isolated bacteria (Simões *et al.*, 2007b, 2008a). Additionally, recent studies into the microbial ecology of DWDS have found that microbial resistance to disinfectants is also affected by microbial community diversity and interspecies relationships (Berry *et al.*, 2006; Simões *et al.*, 2007c, 2010). The ecology of a biofilm is a complex function of prevailing growth conditions, hydrodynamic forces, presence of microbial metabolites and molecules (cell-cell signalling communications) excreted by the microorganisms and dominant microbial inhabitants in the biofilm (Banks and Bryers, 1991; Bryers and Ratner, 2004). The production and detection of bacterial cell-cell signalling molecules have been repeatedly linked to the enhanced development of single and multispecies biofilms (Irie and Parsek, 2008).

Evidence of increased biofilm resistance to conventional disinfection treatments has led to seek several alternative control strategies. These include the use of interspecies interactions as biocontrol strategies (Gram *et al.*, 1999; Mireles *et al.*, 2001; Ammor *et al.*, 2006), bacteriophages (Hughes *et al.*, 1998; Tait *et al.*, 2002; Sillankorva *et al.*, 2004, 2010), enzymes (Meyer 2003; Olsen *et al.*, 2007; Leroy *et al.*, 2008; Lequette *et al.*, 2010), QSI (Rasmussen *et al.*, 2005) and other compounds with potential interference in biofilm formation such as iron chelators (Singh *et al.*, 2002; Banin *et al.*, 2005) and bacteriocin-like substances (Riley, 1998; Messi *et al.*, 2011). However, in order to develop new strategies for preventing biofilm formation, it is necessary to better understand the mechanisms by which different species survive and interact within a biofilm. Such biological mechanisms, alone or as part of synergistic procedures, could provide a new line of efficient biofilm control strategies.

The aim of this study is to understand the effects of metabolite molecules produced by DW-isolated bacteria on single and multispecies biofilm formation and development and to evaluate their potential as biocontrol strategy. With this purpose, bacterial aspects regulating interspecies interactions (planktonic and sessile growth rates, cell-free supernatant antimicrobial activity, and production of QSI, QS molecules and iron chelators) were characterized with the intent to identify bacterial species with biocontrol potential in DWDS. Furthermore, the effects of cell-free supernatants of DW bacteria were studied on single and multispecies biofilm formation by DW-isolated bacteria.

9.2 Material and methods

9.2.1 Bacteria isolation and identification

The bacteria used throughout this work were isolated from a model laboratory DWDS, as described previously in chapter 3 and by Simões *et al.* (2006). Identification tests, by determination of 16S rDNA gene sequence, were performed according to the method described in chapter 4 and by Simões *et al.* (2007a). The assays were performed with six representative DW-isolated bacteria, *Acinetobacter calcoaceticus*, *Burkholderia cepacia*, *Methylobacterium sp.*, *Mycobacterium mucogenicum*, *Sphingomonas capsulata* and *Staphylococcus sp.*, respectively.

9.2.2 Planktonic bacterial cell growth

Bacterial cells were grown overnight in batch culture using 100 ml of R2A (Merck, Portugal) broth, at room temperature (23 ± 2 ºC), under agitation (150 rpm). Cells were harvested by centrifugation (20 min at 13000 g, 4 ºC), washed three times in 0.1 M PBS (pH 7.2) and resuspended in a certain volume of R2A broth necessary to achieve a cellular density of 10^8 cells ml^{-1}.

To obtain the cell-free supernatants, the bacteria were inoculated in 100 ml of R2A broth, and allowed to grow for 10 d, to stabilize the growth phase (Andersson *et al.*, 1998), at room temperature and under agitation. Following the incubation period, cell suspensions were centrifuged (20 min at 13000 g, 4 ºC) and the supernatants were filter-sterilization using 0.2 μm filters (Orange Scientific, USA), subsequently, cell-free supernatants were stored at -4 ºC.

9.2.3 Planktonic and sessile growth rates determination

The growth rates of planktonic and sessile DW-isolated bacteria were determined by assessing their growth by batch culture (planktonic) or by 96-well microtiter plate assay according to the modified microtiter plate test proposed by Stepanović *et al.* (2000), for biofilm growth. In both cases R2A broth was used as growth medium. To promote bacterial planktonic and sessile growth over time, the batch cultures and microtiter plates were incubated aerobically on an orbital shaker, at 150 rpm and room temperature. The bacterial growth over time was performed by the standard plate count method using R2A agar as culture medium. The biofilms in each well of microtiter plate were scraped with a sterile scalpel for 200 μl of sterile sodium phosphate buffer (pH 7). Before serial dilution, biofilm suspensions were vortexed for 2 min and then used to assess colony forming units (CFU). CFUs of planktonic and sessile samples were counted after 7-15 d incubation at room temperature to allow the enumeration of slow-growing bacteria adapted to oligotrophic conditions.

9.2.4 Antimicrobial activity assay

To assess the potential antimicrobial activity of cell-free supernatants, these were inoculated onto lawns of the other bacteria grown overnight in R2A broth, at room

temperature and under agitation. The other bacteria (100 µl) were spread onto R2A agar and air-dried for 30 min. Then 10 µl of each cell-free supernatants was directly applied onto the lawns and left to dry, as described by Kolari *et al.* (2001). After that the plates were incubated at room temperature for 3-5 d and subsequently the presence of inhibitory halos was evaluated. Sterile R2A broth was used as negative control.

9.2.5 Bacterial screening for QSI and AHL production

The screening for QSI and AHL production followed the method described by McLean *et al.* (2004). Test bacteria were streaked onto the center of R2A agar plates and grown overnight at room temperature. Indicator microorganisms were grown overnight in LB broth (*Chromobacterium violaceum* O26 and *C. violaceum* 12472) or LB plus 50 µg ml^{-1} spectinomycin (Sigma) and 4.5 µg ml^{-1} tetracycline (*Agrobacterium tumefaciens* A136). Following overnight growth, the test bacteria were overlaid with 5 ml LB soft agar (full strength LB broth containing 0.5% w v^{-1} agar), cooled to 45 °C, containing 10^6 CFU ml^{-1} of the indicator microorganisms *C. violaceum* ATCC 12472. *P. aeruginosa* PAO-1 was used as a positive control for QSI as its two signal molecules, 3-oxo-dodecanoyl homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl homoserine lactone (C4-HSL), competitively bind and inhibit the receptor for the cognate signal *N*-hexanoyl homoserine lactone (C6-HSL), in both indicator microorganisms. *C. violaceum* ATCC 12472 was used as a negative control as it produces the cognate C6-HSL and would therefore not inhibit its own QS signal. A positive QSI result was indicated by a lack of pigmentation of the indicator microorganism, in the vicinity of the test microorganism.

A bioassay for AHL production was performed in order to detect the type of molecule responsible for QSI. Two biosensor microorganisms, *A. tumefaciens* A136 and *C. violaceum* CVO26, were used directly responding to AHLs. The *A. tumefaciens* biosensor is highly sensitive to a variety of AHL chains ranging from C6 to C14, while *C. violaceum* is unable to synthesise its endogenous C6-HSL inducer, but retains the ability to respond to C4-HSL and C6-HSL. For the bioassay, test bacteria were grown on R2A as described above. Following overnight growth, the bacteria were overlaid with LB soft agar containing 5 µl of overnight cultures of *C. violaceum* CVO26, or the *A. tumefaciens* A136 biosensor and incubated overnight at 30 ºC. Following incubation, 50 µl of X-gal (Sigma) solution (20 mg ml^{-1} in dimethylformamide) was added to the *A. tumefaciens* A136 assay plates and

colour development, due to X-gal hydrolysis, allowed to proceed for 15 min at room temperature. *A. tumefaciens* KYC6, a 3-oxo C8 HSL overproducer, was used as positive control for the *A. tumefaciens* biosensor. *C. violaceum* 31532, was the positive control for the *C. violaceum* CVO26 assay. The biosensor strains themselves were used as negative controls as both strains lack AHL synthase genes. A positive test for AHLs was indicated by a blue coloration from X-gal hydrolysis, in the *A. tumefaciens* biosensor, or by a purple CVO26 pigmentation. Negative tests for AHLs were indicated by a lack of coloration.

9.2.6 Iron chelators production

The screening for iron chelators production was assayed on chrome azurol S (CAS) agar, based on the methodology described by Schwyn and Neilands (1987). A positive result was indicated by the colour change of CAS agar, from dark blue to bright yellow. In liquid medium, those molecules were detected by the CAS assay (Schwyn and Neilands, 1987). Equal volumes of cell-free supernatants and CAS assay solution were mixed and left for 30 min at room temperature. The absorbance at 630 nm (BIO-TEK, Synergy HT, Vermont, USA) was measured with sterile medium and CAS assay solution as blank. A negative value indicated the presence of iron-chelating molecules, such as siderophores.

9.2.7 Single and multispecies biofilm formation with and without cell-free supernatants

Single and multispecies biofilms were developed according to the modified microtiter plate test proposed by Stepanović *et al.* (2000) using R2A broth as growth medium. Single species biofilm formation was performed with the six isolated bacteria and with the bacteria plus cell-free supernatants of other different bacteria. Multispecies biofilms were developed at seven different bacterial combinations: one mixture of all six bacteria and six combinations with a mixture of five distinct bacteria, through a strain exclusion process (biofilm formation in the absence of a specific strain or replacing the specific strain by its cell-free supernatant, obtaining distinct species combinations) (Simões *et al.*, 2008a). For each condition the wells of a sterile 96-well flat tissue culture plates (polystyrene, Orange Scientific, USA) were filled under aseptic conditions with 180 µl of a cell suspension and 20 µl of cell-free supernatant resulting in final concentration of 10^8 cells

ml^{-1}. Multispecies biofilms were developed with equal initial cell densities of each isolate. To promote biofilm formation, plates were incubated aerobically on an orbital shaker, at 150 rpm and room temperature, for 24, 48 and 72 h. The growth medium was carefully discarded and freshly added every 24 h. All experiments were performed in triplicate with at least three repeats. Negative controls were obtained by incubating the wells with R2A broth without adding any bacterial cells. After each biofilm formation period, the content of each well was removed and the wells were washed three times with 250 µl of sterile distilled water to remove reversibly adherent bacteria. Afterwards, the biofilms were analysed in terms of biomass adhered and metabolic activity.

9.2.8 Biomass quantification by CV

The bacterial biofilms in the 96-wells polystyrene microtiter plates were fixed with 250 µl of 98% methanol (Vaz Pereira, Portugal) per well for 15 min. Afterwards, the plates were emptied and left to dry. Then, the fixed bacteria were stained for 5 min with 200 µl of CV (Gram-colour-staining set for microscopy, Merck) per well. Excess stain was rinsed off by placing the plate under running tap water (Stepanović *et al.*, 2000). After the plates were air dried, the dye bound to the adherent cells was resolubilized with 200 µl of 33% (v v^{-1}) glacial acetic acid (Merck, Portugal) per well.

The OD of the obtained solutions were measured at 570 nm using a microtiter plate reader (BIO-TEK, Model Synergy HT) and biofilm mass results are presented as relative percentage according to the following expressions:

For single species biofilms:

$$\text{Relative biofilm mass}\,(\%) = \frac{\text{OD}_{570\text{nm}}\ \text{of biofilms with cell-free supernatant}}{\text{OD}_{570\text{nm}}\ \text{of biofilms without cell-free supernatant}} \times 100$$

For multispecies biofilms:

$$\text{Relative biofilm mass}\,(\%) = \frac{\text{OD}_{570\text{nm}}\ \text{of biofilms with strain exclusion}}{\text{OD}_{570\text{nm}}\ \text{of biofilms with all six bacteria}} \times 100$$

9.2.9 Respiratory activity assessment by XTT

The sodium 3,3'-[1[(phenylamino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) XTT colorimetric method was applied to determine the bacterial activity of the biofilms as described previously by Stevens and Olsen (1993), with some modifications. The combined solution of XTT (Sigma) and PMS (Sigma) was added to each well, in order to obtain a final concentration of 50 μg ml^{-1} of XTT and 10 μg ml^{-1} of PMS. This solution was also added to each well of the 96-wells microtiter plates with bacterial biofilms. Then, the microtiter plates were incubated for 3 h in the dark, at 150 rpm and room temperature. The OD of the formazan supernatant of each well was measured at 490 nm using a microtiter plate reader (BIO-TEK, Model Synergy HT). The biofilm specific respiratory activity was presented as $OD_{490\ nm/570\ nm}$ (biofilm respiratory activity/biofilm mass), according to Simões *et al.* (2007b) and the results are presented as relative percentage according to the following expressions:

For single species biofilms:

$$\text{Relative specific biofilm activity (\%)} = \frac{OD_{\frac{490\ nm}{570\ nm}}\ \text{of biofilms with cell-free supernatant}}{OD_{\frac{490\ nm}{570\ nm}}\ \text{of biofilms without cell-free supernatant}} \times 100$$

For multispecies biofilms:

$$\text{Relative specific biofilm activity (\%)} = \frac{OD_{\frac{490\ nm}{570\ nm}}\ \text{of biofilms with strain exclusion}}{OD_{\frac{490\ nm}{570\ nm}}\ \text{of biofilms with all six bacteria}} \times 100$$

9.2.10 Biofilm classification and statistical analysis

In order to understand the effects of cell-free supernatants on single and multispecies biofilms the values of relative percentage of mass or activity for each biofilm (% B) were compared with the values of relative percentage of biofilm mass and activity for the control experiment (% B_C). For the single species biofilms, B_C is the biofilm without cell-free supernatant, while for multispecies biofilm is the biofilm with all six bacteria. The biofilms were classified as follows: strongly inhibited (− −) – % B ≤ 0.5 Bc (%); weakly inhibited (−) - 0.5 Bc (%) < % B < Bc (%); strongly improved (+ +) - % B ≥ 2 Bc (%); weakly improved

(+) – Bc (%) < % B < 2 Bc (%). For this classification it was considered the average value of the relative percentage of biofilm mass or activity for the three sampling times.

The data were analysed using the statistical program SPSS version 17.0. The mean and standard deviation within samples were calculated for all cases. The data were analyzed by the nonparametric Wilcoxon test based on a confidence level of ≥ 95% ($P < 0.05$ was considered statistically significant).

9.3 Results

9.3.1 Planktonic and biofilm growth rates

Planktonic and biofilm growth was performed with the DW-isolated bacteria to assess growth rates in R2A broth (Table 9.1). *B. cepacia* showed the highest growth rate and *A. calcoaceticus* the smallest for planktonic cells. *Methylobacterium* sp. and *Sph. capsulata* had similar growth rates ($P > 0.05$), and higher than that of *M. mucogenicum* and *Staphylococcus* sp. ($P < 0.05$). Concerning biofilm growth rates, *Methylobacterium* sp. biofilms had the highest growth rate and *M. mucogenicum* biofilms the lowest. *Sph. capsulata* and *Staphylococcus* sp. biofilms had similar growth rates ($P > 0.05$), which were higher than those observed for *A. calcoaceticus* and *B. cepacia* biofilms ($P < 0.05$). Biofilm growth rates were always higher than planktonic for all the bacteria tested ($P < 0.05$).

Table 9.1 Growth rates of planktonic cultures and biofilms

Bacteria	Growth rates (h^{-1})	
	Planktonic	Biofilm
A. calcoaceticus	0.0313 ± 0.008	0.745 ± 0.057
B. cepacia	0.174 ± 0.005	0.772 ± 0.018
Methylobacterium sp.	0.112 ± 0.004	1.003 ± 0.035
M. mucogenicum	0.0757 ± 0.015	0.623 ± 0.070
Sph. capsulata	0.119 ± 0.003	0.851 ± 0.020
Staphylococcus sp.	0.0893 ± 0.014	0.903 ± 0.085

9.3.2 Antimicrobial activity, production of QSI, AHLs and iron chelators

Planktonic studies were performed with the DW-isolated bacteria to assess the antimicrobial activity of cell-free supernatants, and the production of QSI, AHLs and iron chelators (Table 9.2).

Table 9.2 Screening for antimicrobial activity, QSI, AHLs and iron chelators production from DW-isolated bacteria supernatants

Bacteria	Antimicrobial activity	QSI	AHLs		Iron chelators	
			(C4-HSL and C6-HSL)	(C6-HSL-C14-HSL)	CAS agar	CAS assay
A. calcoaceticus	–	–	–	–	–	–
B. cepacia	–	+	+	+	+	+
Methylobacterium sp.	–	+	+	+	+	–
M. mucogenicum	–	–	–	–	–	–
Sph. capsulata	–	+	–	+	–	–
Staphylococcus sp.	–	+	–	–	–	–

– Not detected; + Detected.

The assays of the antimicrobial activity with all the supernatants have shown no effects on bacterial growth. The screening of QSI and AHLs production showed that *B. cepacia*, *Methylobacterium* sp., *Sph. capsulata*, and *Staphylococcus* sp. produced QSI molecules. Only for *Staphylococcus* sp. QSI were not related with AHLs. This was expected, due to the peptide-like molecules involved in Gram-positive bacteria QS events, not detected by the methodology used. Table 9.2 also shows that only *B. cepacia* and *Methylobacterium* sp. produced iron-chelating molecules, such as siderophores. However, only *B. cepacia* was able to produce extracellular iron-chelating molecules.

9.3.3 Single species biofilm formation with and without cell-free supernatants

In order to study the effects of cell-free supernatants of each bacterium in single species biofilm formation ability and specific respiratory activity of the several DW-isolated bacteria, the standard 96-well microtiter plate technique with CV and XTT staining was used to characterize the biofilms. To better understand the function of cell-free supernatants in single species biofilm formation, the relative percentages of biofilm mass (Figure 9.1) and specific respiratory activity (Figure 9.2) were calculated over time and compared with

each single species biofilm. Additionally, the single species biofilms were classified as strongly/weakly inhibited or strongly/weakly improved by the presence of cell-free supernatants (Table 9.3).

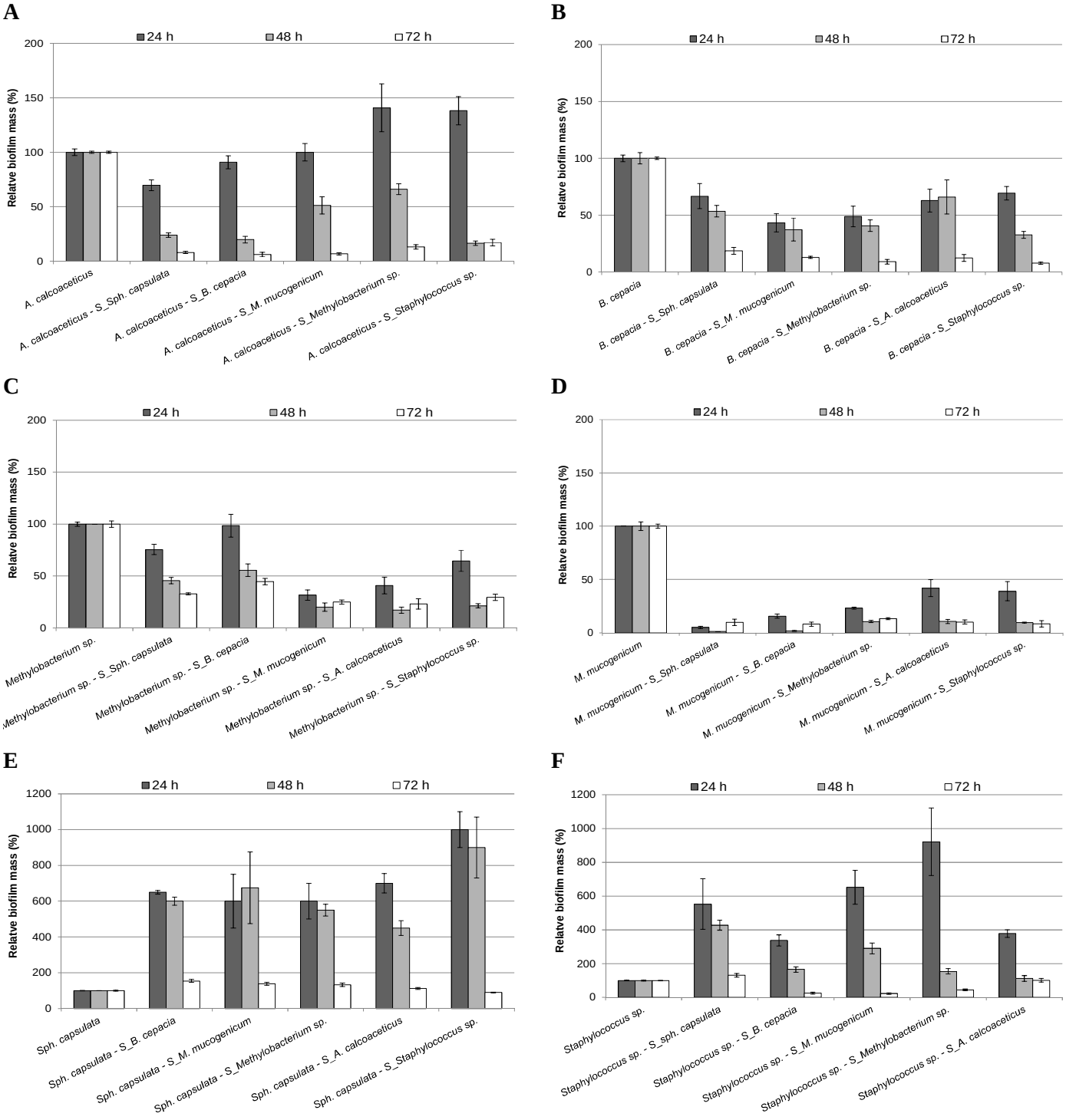

Figure 9.1 Relative percentage of biofilm mass for *A. calcoaceticus* (A), *B. cepacia* (B), *Methylobacterium* sp. (C), *M. mucogenicum* (D), *Sph. capsulata* (E) and *Staphylococcus* sp. (F) single species biofilms with cell-free supernatants from the partner DW bacteria, at

24, 48 and 72 h of biofilm formation. The means ± standard deviations for at least three replicates are illustrated.

In general, it is possible to observe an inversely proportional relationship of time–biomass formation for the several single species biofilms with cell-free supernatants (Figure 9.1). *A. calcoaceticus* (Figure 9.1A), *B. cepacia* (Figure 9.1B), *Methylobacterium* sp. (Figure 9.1C) and *M. mucogenicum* (Figure 9.1D) biofilms were inhibited by the cell-free supernatants from the other bacteria. Their biomass was lower than that of single species biofilms without cell-free supernatants ($P < 0.05$), with exception of *A. calcoaceticus* biofilms (24 h) with *Methylobacterium* sp. and *Staphylococcus* sp. cell-free supernatants. *M. mucogenicum* single biofilms were the most affected by the cell-free supernatants from the other bacteria, followed by *B. cepacia* and *Methylobacterium* biofilms ($P < 0.05$). *A. calcoaceticus* biofilms seems to be the least affected. On the other hand, *Sph. capsulata* (Figure 9.1E) and *Staphylococcus* sp. (Figure 9.1F) biofilms were potentiated in the presence of cell-free supernatants from the other bacteria ($P < 0.05$). The biofilm mass was higher than the single species biofilms without cell-free supernatants, with the exception of the *Sph. capsulata* biofilms (72 h) with *Staphylococcus* sp. cell-free supernatant, *Staphylococcus* sp. biofilms (72 h) with *B. cepacia*, *M. mucogenicum* and *Methylobacterium* sp. cell-free supernatants. *Sph. capsulata* (Figure 9.1E) single biofilms had the highest biomass increase in the presence of cell-free supernatants from the other DW-isolated bacteria.

Concerning the percentage of specific respiratory activity (Figure 9.2) it was verified a general decrease over time for *Methylobacterium* sp. and *M. mucogenicum* single species biofilms in the presence of cell-free supernatants; however, the values were mostly higher than for the control experiments. The other bacteria (*A. calcoaceticus*, *B. cepacia*, *Sph. capsulata* and *Staphylococcus* sp.) formed single biofilms in the presence of cell-free supernatants with a general increase of metabolic activity over time. *Sph. capsulata* (Figure 9.2E) was the only single species biofilm that activity was significantly inhibited by the cell-free supernatants from the different bacteria when comparing with the control experiments ($P < 0.05$). The other single biofilms had a variable behaviour in the presence of cell-free supernatants. *Staphylococcus* sp. single biofilms were the subsequent biofilms more affected by the presence of cell-free supernatants, following by *Methylobacterium* sp. single biofilms. *B. cepacia* and *A. calcoaceticus* single biofilms were affected in a lower extent and

M. mucogenicum biofilms were the least affected by the presence of cell-free supernatants from other bacteria.

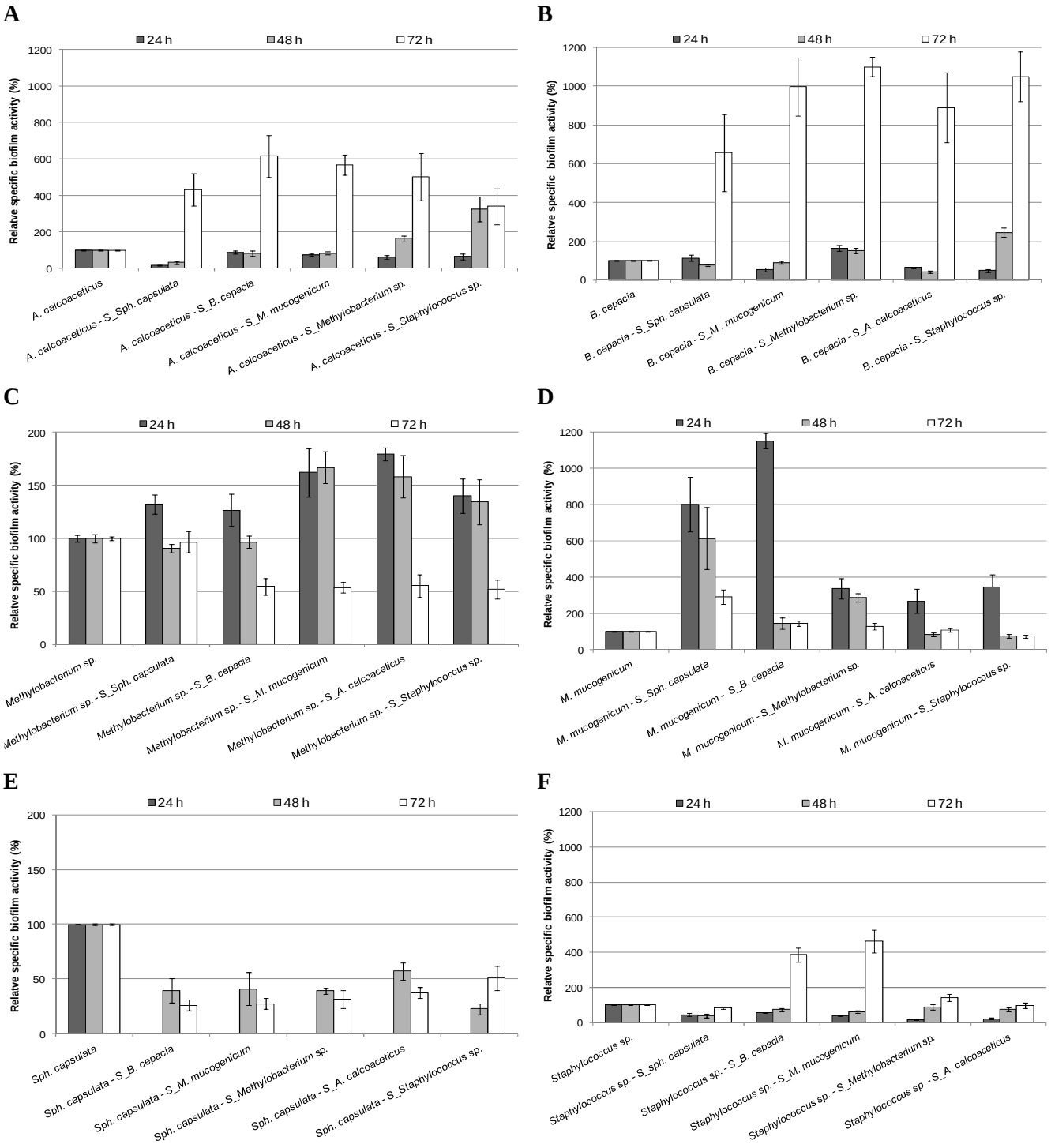

Figure 9.2 Relative percentage of specific biofilm activity for *A. calcoaceticus* (A), *B. cepacia* (B), *Methylobacterium* sp. (C), *M. mucogenicum* (D), *Sph. capsulata* (E) and *Staphylococcus* sp. (F) single species biofilms with cell-free supernatants from the partner

DW bacteria, at 24, 48 and 72 h of biofilm formation. The means ± standard deviations for at least three replicates are illustrated.

Comparing the percentage of relative biofilm mass and specific activity (Figure 9.1 and 9.2) for each single species biofilms it was verified that those biofilms most affected by cell-free supernatants in terms of mass were the least affected in terms of metabolic activity. For instance, *M. mucogenicum* single biofilms were the most inhibited in terms of mass by the cell-free supernatants from the other bacteria; however, these biofilms were the most metabolically actives, i.e. the cell-free supernatants potentiated biofilm activity. This finding was also verified by the results of the biofilm classification of single species biofilms present in Table 9.3. According to this Table there are contradictory results between the mass and specific metabolic activity for all the single species biofilms, except for *Methylobacterium* sp. single species biofilm with *B. cepacia* cell-free supernatant, and *Staphylococcus* sp. biofilms with *B. cepacia* and *M. mucogenicum* cell-free supernatants. For those biofilms the presence of cell-free supernatant lead a simultaneous decrease or increase on biofilm formation and specific metabolic activity, respectively. As regards the biofilm classification of Table 9.3, concerning the mass results, cell-free supernatants inhibited all single species biofilms, except those *Sph. capsulata* and *Staphylococcus* sp. Those were increased in the presence of all cell-free supernatants. All cell-free supernatants caused strong mass increase on *Sph. capsulata* biofilms. However, on *Staphylococcus* sp. biofilms, *A. calcoaceticus* and *B. cepacia* cell-free supernatants only led a weak increase on biofilm mass and the other supernatants led a strong increase on biofilm formation. The biofilms inhibited by cell-free supernatants were, for the most part, strongly inhibited. *B. cepacia* and *M. mucogenicum* biofilms were the most affected by cell-free supernatants (strongly inhibited for all the situations), while *Methylobacterium* sp. and *A calcoaceticus* biofilms were the least affected.

Table 9.3 Effects of cell-free supernatants on mass and metabolic activity of single species biofilms of DW-isolated bacteria

Single species biofilms \ Cell-free supernatants	*A. calcoaceticus*		*B. cepacia*		*Methylobacterium* sp.		*M. mucogenicum*		*Sph. capsulata*		*Staphylococcus* sp.	
	Mass	Activity	Mass	Activity	Mass	Activity	Mass	Activity	Mass	Activity	Mass	Activity
A. calcoaceticus			– –	+ +	–	+ +	–	+ +	– –	+	–	+ +
B. cepacia	– –	+ +			– –	+ +	– –	+ +	– –	+ +	– –	+ +
Methylobacterium sp.	– –	+	–	–			– –	+	–	+	– –	+
M. mucogenicum	– –	+	– –	+ +	– –	+ +			– –	+ +	– –	+
Sph. capsulata	+ +	– –	+ +	– –	+ +	– –	+ +	– –			+ +	– –
Staphylococcus sp.	+	–	+	+	+ +	–	+ +	+	+ +	–		

– – Strong biofilm inhibition; – Weak biofilm inhibition.

+ + Strong biofilm increase; + Weak biofilm increase.

9.3.4 Multispecies biofilm formation with and without cell-free supernatants

To better understand the function of each bacterium and/or their metabolites on multispecies biofilm formation, the relative percentage of biofilm (mass and activity) was calculated for multispecies biofilm with and without cell-free supernatants (Figure 9.3). Moreover, similarly to the single species biofilms and with the purpose to better understand the effects of each cell-free supernatant/bacteria on multispecies biofilm mass and specific metabolic activity, biofilms were classified as strongly/weakly inhibited or strongly/weakly improved by the presence of cell-free supernatants or the absence of one specific bacterium (Table 9.4).

The mass of multispecies biofilms without cell-free supernatants increased over time for all the situations, except for multispecies biofilms without *A. calcoaceticus* (Figure 9.3A). *M. mucogenicum* was the only bacterium that, when not present, leads to a relative increase of biofilm mass over time comparatively to biofilm formation with all six bacteria. The remaining bacteria reduced biofilm formation. The decrease of mass formation was less significant ($P > 0.05$) for biofilms in the absence of *Sph. capsulata* (24 and 48 h) and *Staphylococcus* sp. (72 h), and more significant ($P < 0.05$) in the absence of *B. cepacia* (24 h) and *A. calcoaceticus* (48 and 72 h). Nevertheless, even if the relative biofilm formation decreased for five of the six strain exclusion scenarios, it was only significant ($P < 0.05$) and decreased over time ($P < 0.05$) for biofilms without *A. calcoaceticus*. According to Table 9.4 only biofilms in the absence of *A. calcoaceticus* were strongly inhibited, the other biofilms were weakly inhibited, except multispecies biofilms without *M. mucogenicum* that were weakly improved.

All six strain exclusion scenarios increased biofilm activity (Figure 9.3B) comparatively to multispecies biofilms with all six bacteria, except biofilms without *Sph. capsulata* and *M. mucogenicum* (72 h). The decrease of activity was only significant for biofilms in the absence of *M. mucogenicum* (Table 9.4). The increase of biofilm activity was less significant for biofilms in the absence of *M. mucogenicum* (24 and 48 h) and *Staphylococcus* sp. (72 h), and more significant in the absence of *B. cepacia* (24 h) and *A. calcoaceticus* (48 and 72 h). However, even if the relative biofilm activities increase for all strain exclusion scenarios, it was only significant and increase over time for biofilms without *A. calcoaceticus* ($P < 0.05$). Biofilm classification (in terms of activity) shows that

the majority of biofilms were strongly (without *B. cepacia* and *A. calcoaceticus*) or weakly (without *Sph. capsulata*, *Methylobacterium* sp. and *Staphylococcus* sp.) improved (Table 9.4). The biofilms without *M. mucogenicum* were weakly inhibited.

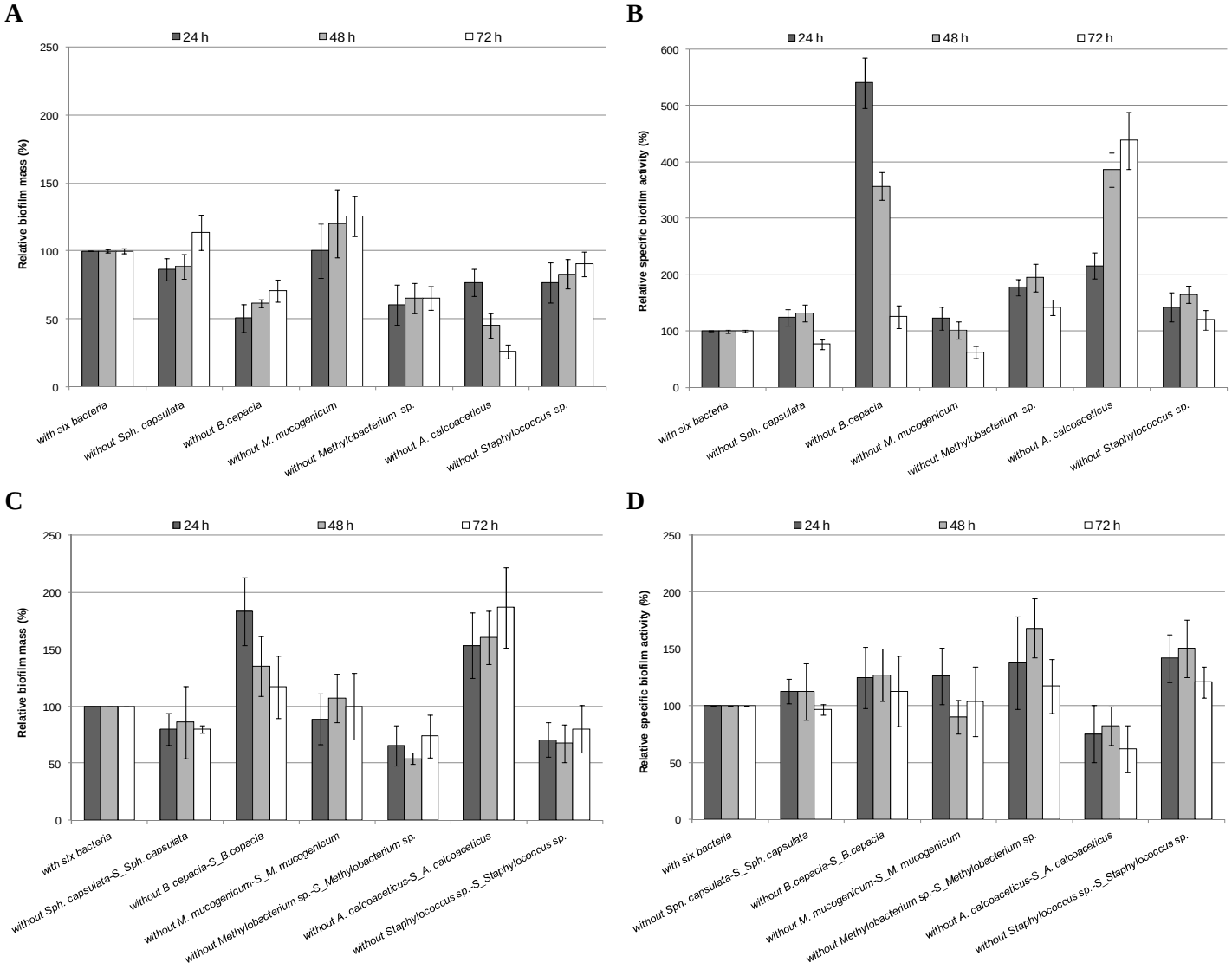

Figure 9.3 Relative percentage of biofilm mass (A and C) or specific biofilm metabolic activity (B and D) for multispecies biofilms with (C and D) and without (A and B) cell-free supernatants from the partner DW bacteria, at 24, 48 and 72 h of biofilm formation. The means ± standard deviations for at least three replicates are illustrated.

Analysing the multispecies biofilms with cell-free supernatants (Figure 9.3C), biofilm mass tends to increase over time, except for biofilms with *B. cepacia* cell-free supernatants. Multispecies biofilms with *B. cepacia*, *A. calcoaceticus* and *M. mucogenicum* (48 and 72 h) cell-free supernatants leads to a relative increase of biofilm mass

comparatively to biofilm formation with the six bacteria. The other cell-free supernatants reduced biofilm mass. The decrease of biofilm mass formation was less significant for biofilms with *M. mucogenicum* (24 h), *Sph. capsulata* (48h) and *Staphylococcus* sp. (72 h) cell-free supernatants, and more significant with *Methylobacterium* sp. cell-free supernatants (24, 48, and 72 h). On the other hand, the increase of biomass was less significant for biofilms with *A. calcoaceticus* (24 h) and *M. mucogenicum* (48 and 72 h) cell-free supernatants, and more significant in the presence of *B. cepacia* (24 h) and *A. calcoaceticus* (48 and 72 h) cell-free supernatants. According to Table 9.4 multispecies biofilms with cell-free supernatants were weakly inhibited for most of the situations, except for biofilms with *B. cepacia* and *A. calcoaceticus* cell-free supernatants (weakly improved).

The results of multispecies biofilm metabolic activity with cell-free supernatants (Figure 9.3D) shows that five of the six strain exclusion scenarios increased activity (considering the average values of three sampling times). Only multispecies biofilms in the presence of *A. calcoaceticus* cell-free supernatants reduced the biofilm activity over time comparatively to the multispecies biofilms with all six bacteria. The increase of biofilm activity was less significant for biofilms with the cell-free supernatants of *Sph. capsulata* (24 and 48 h) and *M. mucogenicum* (72 h), and more significant in the presence of *Staphylococcus* sp. (24 and 72 h) and *Methylobacterium* sp. (48 h) cell-free supernatants. According to Table 9.4 for biofilm activity, the presence of all cell-free supernatants weakly improved biofilm metabolic activity, except the *A calcoaceticus* cell-free supernatant that weakly inhibited biofilm activity.

Table 9.4 Effects of cell-free supernatants on mass and metabolic activity of multispecies biofilms of DW-isolated bacteria

Cell-free supernatants / Multispecies biofilms	Without		With	
	Mass	Activity	Mass	Activity
Without *Sph. capsulata*	−	+	−	+
Without *B. cepacia*	−	+ +	+	+
Without *M. mucogenicum*	+	−	−	+
Without *Methylobacterium* sp.	−	+	−	+
Without *A. calcoaceticus*	− −	+ +	+	−
Without *Staphylococcus* sp.	−	+	−	+

− − Strong biofilm inhibition; − Weak biofilm inhibition.
+ + Strong biofilm increase; + Weak biofilm increase.

Biofilm mass and activity results are inversely related. Similarly to single species biofilms, contradictory results between biofilm mass and specific metabolic activity were verified for all multispecies biofilms tested, except for multispecies biofilms with *B. cepacia* cell-free supernatant (Table 9.4).

The comparison between multispecies biofilms with and without cell-free supernatants (Figure 9.3A and C) shows that the absence of *B. cepacia* and *A. calcoaceticus* decreased biofilm formation ability (Figure 9.3A). However, if the biofilms were formed with the cell-free supernatants of those bacteria (Figure 9.3C), it was verified a high biofilm productivity. This biomass increase is inversely proportional to biofilm reduction caused by lacking those species in the bacterial consorcium. On the other hand, *Sph. capsulata*, *M. mucogenicum* and *Staphylococcus* sp. cell-free supernatants promoted a slight reduction of biofilm formation comparatively to those without cell-free supernatants. Nonetheless, the absence of *M. mucogenicum* from multispecies biofilms promoted a small increase of biofilm formation. The presence of its cell-free supernatant promoted a slight reduction of biofilm formation. *Methylobacterium* sp. cell-free supernatants did not promoted significant changes on multispecies biofilm formation.

Regarding multispecies biofilm activity (Figure 9.3B and D) the presence of *Sph. capsulta* and *Staphylococcus sp.* cell-free supernatants had no significant impact on biofilm activity ($P > 0.05$). *M. mucogenicum* cell-free supernatant improved slightly biofilm activity. In contrast, *B. cepacia*, *Methylobacterium* sp. and *A. calcoaceticus* cell-free supernatants caused a significant reduction on the activity of multispecies biofilms. This reduction was more pronounced for biofilms with *B. cepacia* and *A. calcoaceticus* cell-free supernatants ($P < 0.05$) and less significant for biofilms with *Methylobacterium* sp. ($P > 0.05$). However, only in multispecies biofilm without *A. calcoaceticus*, its cell-free supernatant caused a strong inhibition of metabolic activity, comparatively to multispecies biofilms composed by the six bacteria ($P < 0.05$).

Comparing the results obtained for multispecies biofilms classification with and without cell-free supernatants (Table 9.4), it was verified that biofilms without *Sph. capsulata*, *Methylobacterium* sp. and *Staphylococcus* sp. provided similar results ($P > 0.05$). Those biofilms had similar biomass inhibition and metabolic activity increase whether in the presence or absence of cell-free supernatants. *B. cepacia* and *A. calcoaceticus* cell-free supernatants were important for multispecies biofilm formation, because their presence

improved biofilm formation. Conversely, *M. mucogenicum* cell-free supernatants inhibited multispecies biofilm formation.

9.4 Discussion

Control of microbial growth is essential in many environments, where wet or moist surfaces provide favorable conditions for microbial proliferation and biofilm formation (Simões *et al.*, 2009). In the industrialized world DW that arrives to the consumer goes through a complex process of treatment and disinfection. However, during distribution their quality may deteriorate and becomes harmful to human health. The formation and presence of biofilms in DWDS have been repeatedly reported and their undesirable effects in the quality of distributed water are well known (Momba *et al.*, 1999; Paris *et al.*, 2009). DW biofilms are complex communities which host a wide variety of microorganisms, well adapted to oligotrophic conditions and often grow in the presence of disinfectant (Berry *et al.*, 2006). These biofilms can harbour, protect and allow the proliferation of several opportunistic and pathogenic bacteria. For that reason, biofilm control is crucial. However, biofilm resistance to conventional disinfection promotes the constant search for alternative control strategies. This study has impact for understanding about the biological mechanisms by which diverse species survive and interact in DW biofilm communities and potentially identify new biofilm control strategies that will ensure safe and high-quality DW. To our knowledge this is the first study that investigates the effects of interspecies interactions and production of metabolite molecules from DW-isolated bacteria on their biofilm formation/control and behaviour.

The bacteria used in this study are recognized as problematic opportunistic bacteria with the potential to cause public health problems (Bifulco *et al.*, 1989; Rusin *et al.*, 1997; Szewzyk *et al.*, 2000; Conway *et al.*, 2002; Pavlov *et al.*, 2004; Stelma *et al.*, 2004). Similarly to other studies, single and multispecies biofilms were developed in polystyrene microtiter plates that is the most frequently used bioreactor system for studying biofilm formation, providing reliable comparative data (Djordjevic *et al.*, 2002; Sandberg *et al.*, 2008; Cotter *et al.*, 2009). Microtiter plates can be used as a rapid and simple method to screen the effects of cell-free supernatants on single and multispecies biofilm formation by DW-isolated bacteria. Polystyrene was used only as a model surface; however, it has

physico-chemical surface properties similar to those of other materials commonly used in DWDS such as stainless steel and polyvinylchloride (Simões *et al.*, 2007a). The selected assay, based on CV staining, is a well-known method for staining biofilms produced by several Gram-positive and Gram-negative bacterial strains (O'Toole and Kolter, 1998; Stepanović *et al.*, 2000; Djordjevic *et al.*, 2002). CV method is a simple protocol of biofilm mass staining that uses reagents that are inexpensive and easily available. This method provides reliable results, making it an attractive screening assay for small-scale laboratories (Sandberg *et al.*, 2008). The biofilm metabolic activity was measured by XTT staining assay. The XTT assay has been used extensively for the quantification of metabolically active cells in biofilms (Simões *et al.*, 2010).

The mechanisms that control microbial interactions in multispecies biofilms are not fully understood. In our previous publication on the study of the biofilm interactions between DW-isolated bacteria, it was possible to identify several types of microbial interactions: synergistic, antagonistic and neutral interactions (Simões *et al.*, 2007b). However, in this work it was not possible to understand the biological mechanisms involved in the relationships between these bacteria. The existence of multiple interactions or even the simple production of a metabolite can interfere (inhibit/potentiate) with the development of structurally organized biofilms (Simões *et al.*, 2007b). Chemical substances secreted by one species of microorganisms can significantly influence the colonization of the other species (Holmström *et al.*, 2002). To determine which factors may influence the interaction of multispecies biofilms, the DW-isolated bacteria were characterized in terms of key characteristics (planktonic and sessile growth rates, cell-free supernatants antimicrobial activity, production of QSI and iron chelators). These features are recognized as important factors regulating interspecies interactions and multispecies biofilm formation (Daniels *et al.*, 2004; Banin *et al.*, 2005; Kolmos *et al.*, 2005; McLean *et al.*, 2004; Moons *et al.*, 2006). According to existing descriptions of multispecies biofilm population dynamics, the faster growing bacteria should out-compete those that grow more slowly (Wanner and Gujer, 1986; Banks and Bryers, 1991). However, many studies have shown slower-growing organisms to coexist with or even out-compete the faster-growing organisms in multispecies biofilms (Christensen *et al.*, 2002; Komlos *et al.*, 2005). The production of antimicrobial compounds, including toxins, bacteriolytic enzymes, bacteriophages, biosurfactants, antibiotics and bacteriocins seems to be a generic phenomenon for most bacteria (Riley, 1998). The production of a bacteriocin could give an organism a competitive advantage

when interacting with other microbes, both in gaining a foothold in a new environment and also in preventing the colonization of a potential competitor into a pre-established biofilm (Tait and Sutherland, 2002). Furthermore, many bacteria are also capable of synthesizing and excreting biosurfactants with anti-adhesive properties (Desai and Banat, 1997; Nitschke and Costa, 2007). QS, a cell density-related communication mode between one or more species, is a significant factor adding complexity to the interactions between biofilm bacteria. It is also known to influence bacterial community development in aquatic biofilms (McLean *et al.*, 2005; Dobretsov *et al.*, 2009). QS plays a role in cell attachment and detachment from biofilms (Davies *et al.*, 1998; Donlan, 2002; Daniels *et al.*, 2004). According to Moons *et al.* (2006) interference with QS may reduce the ability of biofilm bacteria to exclude competitors and, in this way, cause a shift in the natural biofilm composition. Production of iron chelators such as siderophores is a virulence factor in many microorganisms, acting as biocontrol molecules (Gram *et al.*, 1999). Iron binding molecules have been found to have a bacteriostatic activity. Siderophores dissolve iron ions, essential to microbial survival, microbial interactions and biofilm formation, and soluble Fe^{3+} complexes can be taken up by active transport mechanisms (Banin *et al.*, 2005). Antagonistic interactions were described for a *Bacillus cereus* and *Pseudomonas fluorescens* in planktonic and biofilm systems due to the production of siderophores by *P. fluorescens* (Simões *et al.*, 2008b).

This study shows that *B. cepacia* and *Methylobacterium* sp. had the highest growth rate on planktonic and sessile state respectively, produced QSI (AHL-related molecules) and iron chelators. These bacteria had apparently the best competitive advantage in multispecies biofilms. *Sph. capsulata* also presented a considerably high growth rate (planktonic and sessile), produced QSI, but not iron chelators. *Staphylococcus* sp. had lower growth rates, and had the ability to produce QSI but not iron chelators. On the other hand, *A. calcoaceticus* and *M. mucogenicum* had the lowest growth rate on planktonic and sessile state respectively, and had not the ability to produce QSI and iron chelators. These bacteria had apparently the worst competitive advantage in the biofilm consortium. All the cell-free supernatants had no antimicrobial activities.

In this study, for nearly all scenarios it was verified contradictory results between mass and metabolic activity. The specific metabolic activity was determined to be inversely related to biofilm mass increase. Similar results were obtained in our previous study (Simões *et al.*, 2007b). This could be explained by the fact that following microbial

attachment, the formation of a complex extracellular polymeric matrix increased the non-metabolically active biofilm mass, consequently, decreasing the specific respiratory activity (Simões *et al.*, 2005).

The assessment of the effects of metabolite molecules on single species biofilms allowed to identify four biofilms (*A. calcoaceticus*, *B. cepacia*, *Methylobacterium* sp. and *M. mucogenicum*) which were inhibited and two biofilms which were improved (*Sph. capsulata* and *Staphylococcus* sp.) by the metabolite molecules produced by DW-isolated bacteria. This suggests that all bacterial metabolite molecules had potential biocontrol properties in four of six single species biofilms. *M. mucogenicum* and *B. cepacia* biofilms were strongly inhibited by all cell-free supernatants. These biofilms are the most susceptible to metabolites produced by different bacteria. On the other hand, *A. calcoaceticus* biofilms were the least affected biofilms. The bacteria that produced metabolite molecules more inhibitory to single species biofilm formation were *Sph. capsulata* (on *M. mucogenicum* and *A. calcoaceticus* biofilms) and *M. mucogenicum* (on *B. cepacia* and *Methylobacterium* sp. biofilms). *Sph. capsulata* biofilm formation was strongly improved by the presence of all cell-free supernatants, but *Staphylococcus* sp. cell-free supernatant promoted the most significant increase in biofilm formation. For *Staphylococcus* biofilms, the cell-free supernatant which more increased biofilm formation was these from *Methylobacterium* sp.

According to Shank and Kolter (2009), many microorganisms can grow better in combination with others or in the presence of their partner's diffusible compounds. Several species can coexist in biofilms being its behaviour the sum of synergistic and antagonistic interactions, as they can produce metabolites that can interfere negatively or positively with growth and biofilm formation. Biofilms in DWDS are constituted by several different bacterial species, so it is important to understand the role of each species and/or their metabolite molecules, produced during growth, in the community. The present results show that all the bacteria except *M. mucogenicum* had an important role in multispecies biofilms, because their absences caused reduction on biofilm formation potential. However, the absence of *A. calcoaceticus* caused the highest reduction. The absence of *M. mucogenicum* improved biofilm formation. Concerning the effects of metabolite molecules produced by DW-isolated bacteria on multispecies biofilms, it was possible to remark that, the presence of *A. calcoaceticus* and *B. cepacia* cell-free supernatants increased biofilm formation. On the other hand, the presence of *Sph. capsulata*, *M. mucogenicum*, *Methylobacterium* sp. and *Staphylococcus* sp. cell-free supernatants decreased multispecies biofilm formation

(compared with the control experiment – biofilms with all bacteria). However, for the comparison of multispecies biofilms with and without cell-free supernatants, *Sph. capsulata*, *M. mucogenicum* and *Staphylococcus* sp. cell-free supernatants lead only a slight reduction of multispecies biofilm formation. The metabolite molecules produced by these bacteria have biocontrol properties. *M. mucogenicum* cell-free supernatant promoted the most significant biocontrol potential. The presence of *Methylobacterium* sp. cell-free supernatant had no significant changes in multispecies biofilm formation. The metabolite molecules produced by this bacterium are not apparently important in biofilm community. On the other hand, *A. calcoaceticus* and *B. cepacia* metabolite molecules had a significant role in multispecies biofilm community; their presence improved significantly biofilm formation.

Despite *A. calcoaceticus* had the lowest planktonic growth rate, one of the lowest biofilm growth rates, and was non-producer of QSI and iron chelators, this bacterium formed single species biofilms which were the least susceptible to microbial metabolites produced by the other DW-isolated bacteria and shown important features in the multispecies biofilm consortium. In these biofilms, the absence of *A. calcoaceticus* caused a significant reduction of biofilm mass. This could be due to the ability of this bacterium to coaggregate. In a previous work (Simões *et al.*, 2008a), it was verified that this bacterium coaggregated with almost all the other bacteria, and its presence in a multispecies community represented a colonization advantage. This bacterium may facilitate the association of the other species that do not coaggregate directly with each other, increasing the opportunity for metabolic cooperation. Min *et al.* (2010) also refer that coaggregation enhance biofilm development between freshwater bacteria. On the other hand, the metabolite molecules produced by this bacterium improved significantly the multispecies biofilm formation, probably due to the production of extracellular proteins and polysaccharides involved in coaggregation. The absence of *B. cepacia* in multispecies biofilms also caused significant biofilm mass reduction, but the presence of their metabolite molecules also improved significantly multispecies biofilm formation. However, this fact could be related with the ability of this bacterium to produce QSI and iron chelators. For *M. mucogenicum* like for *A. calcoaceticus*, it was not detected QSI and iron chelators production. This bacterium had also low growth rates, however, formed single biofilms which were the most affected by the microbial metabolites produced by the other DW bacteria. In multispecies biofilms, its absence improved biofilm formation, but their metabolite molecules caused the most significant biocontrol potential. In our previous work

(Simões *et al.*, 2007b), it was verified that *M. mucogenicum* established only antagonistic (with *Sph. capsulata* and with *Staphylococcus* sp.) or neutral (with *A. calcoaceticus* and with *Methylobacterium* sp.) interactions between some DW-isolated bacteria. The reduction of multispecies biofilm formation caused by the absence of *Methylobacterium* sp. seems not related with the metabolite molecules produced by this bacterium, despite QSI, AHL and iron chelators had been detected. Biofilm mass of multispecies biofilms with and without *Methylobacterium* sp. cell-free supernatants had similar values. This fact could be related with the high biofilm growth rate obtained by this bacterium and their ability to produce biofilms with high cell densities (Simões *et al.*, 2010).

In conclusion, although so far, no studies have investigated how the microbial interactions and the production of metabolite molecules can contribute to the shaping of multispecies biofilms. This study allows to better understand the role of bacterial interactions and metabolites produced by DW-isolated bacteria in biofilm formation and development. The identification of bacterial species which have biocontrol potential (*M. mucogenicum*) or have a significant role in development and maintenance of the DW consortium (*A. calcoaceticus* and *B. cepacia*), may provide new findings important for improving successful control of biofilms in DWDS, in order to obtain high water quality for the consumer.

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CHAPTER 10 CONCLUDING REMARKS AND PERSPECTIVES FOR FURTHER RESEARCH

This chapter presents the general conclusions of this thesis and some suggestions for further research in the scope of this thesis are given.

10.1 General conclusions

Safe DW is essential for human health. Water industries and governments over the world are working together in order to improve DW quality through the effective treatment, monitoring of its physicochemical and microbiological properties, and the design and the operational management of the distribution networks. Although the DW is strictly monitored in developed countries, waterborne outbreaks are still being reported due to microbial contamination. Biofilms contribute notoriously to these events, creating a protective and nutritional reservoir for pathogens growth and survival. DW biofilms constitute the major microbial problem in DWDS that most contributes to the deterioration of water quality that reaches the consumer`s tap. However, their elimination from DWDS is almost impossible, but several factors can be manipulated in order to prevent and control their growth.

The aim of this thesis was to gain deeper insights into the biological and ecological mechanisms involved in biofilm formation in DWDS, with intent to control and prevent their formation. In order to achieve these objectives several aspects were studied throughout this thesis, namely: the effects of hydrodynamic conditions and support material on monitoring of biofilm formation by DW autochthonous bacteria using bioreactors; the influence of bacterial and support material surface properties in adhesion and biofilm formation; assessment of coaggregation abilities by DW-isolated bacteria and their importance on biofilm formation; study of bacterial interspecies interactions involved in biofilm formation; study on single and multispecies biofilm control with SHCand microbial metabolites.

The main conclusions that can be extracted from the work presented in this thesis are the following:

Flow cell and Propella^{TM} reactors allowed the formation of biofilms from DW bacteria on PVC and SS316 under turbulent and laminar flow conditions. The numbers of total and cultivable bacteria in turbulent flow-generated biofilms were similar in both bioreactors, regardless the adhesion surface tested. Under laminar flow, the Propella™ bioreactor allowed the formation of steady-state biofilms with a higher number of total and

cultivable bacteria than those from the flow cell system. A higher number of total and cultivable cells were found on PVC surfaces comparatively to SS when biofilms were formed using the flow cell system. Biofilm formation on PVC and SS was similar in the Propella™ system for both flow regimes. More than 20 distinct cultivable bacteria were isolated from this system.

All selected DW-isolated bacteria and support materials commonly used in DWDS were classified regarding their physicochemical surface properties as hydrophilic and hydrophobic, respectively. SS304, copper, PP, PE and silicone thermodynamically favoured adhesion for the majority of the tested strains, whilst adhesion was generally less thermodynamically favourable for SS316, PVC and glass. Analysis of adhesion demonstrate that in addition to physicochemical surface properties of bacterium and substratum, biological characteristics and mechanisms (microbial surface structures, production of extracellular polymeric substances) are involved in early adhesion processes and may play a determinant role on the bacterial adherence ability. Therefore, the study of adhesion ability only based on physicochemical properties and thermodynamic theory do not provides accurate and reliable results. Furthermore, this work also suggests that strongly adherent bacteria (*A. calcoaceticus*) may play a determinant role in primary colonization of surfaces and possibly on the initial establishment of multispecies biofilms in the real environment.

The studies of adhesion and biofilm formation to PS allow to conclude that the adhesion ability (both thermodynamic prediction and adhesion assays) is correlated with biofilm formation ability only in the early stages of biofilm formation (24 h). For longer periods (48 and 72 h) some bacteria classified as non-adherent produced large amounts of matured biofilms. Initial adhesion do not predict the ability of the DW-isolated bacteria to form a mature biofilm, suggesting that other events such as phenotypic and genetic switch during biofilm development and the production of EPS may play a significant role on biofilm formation and differentiation. *A. calcoaceticus*, *Methylobacterium* sp. and *M. mucogenicum* were those bacteria with the ability to produce more biofilm.

Studies on intergeneric coaggregation demonstrate that only *A. calcoaceticus* autoaggregated and coaggregated with four of the five tested bacteria (exception of *Methylobacterium* sp.). These cell-cell adhesion mechanisms were mediated by lectin-saccharide interactions. *A. calcoaceticus* exhibited a putative bridging function in multispecies biofilm formation, being their presence in biofilms a colonization advantage.

Evidences of synergy/cooperation in dual species biofilm formation were found for *Sphingomonas capsulata* and *Burkholderia cepacia, Sph. capsulata* and *Staphylococcus* sp., and *B. cepacia and Acinetobacter calcoaceticus*; antagonism between *Sph. capsulata and M. mucogenicum, Sph. capsulata and A. calcoaceticus,* and *M. mucogenicum-Staphylococcus* sp. A neutral interaction was found for *Methylobacterium* sp.-*M. mucogenicum, Sph. capsulata-Staphylococcus* sp, *M. mucogenicum-A. calcoaceticus* and *Methylobacterium* sp.-*A. calcoaceticus*. *B. cepacia* had the highest growth rate and motility, and produced QSI. Other bacteria producing QSI were *Methylobacterium* sp., *Sph. capsulata* and *Staphylococcus* sp. Only for *Sph. capsulata-M. mucogenicum, Sph. capsulata-A. calcoaceticus* and *M. mucogenicum-Staphylococcus* sp., dual biofilm formation seems to be regulated by the QSI produced by *Sph. capsulata* and *Staphylococcus* sp. and by the increased growth rate of *Sph. capsulata*.

Methylobacterium sp. and *M. mucogenicum* single species biofilms had the highest resistance to SHC, while *Staphylocooccus* sp. and *A. calcoaceticus* formed the most susceptible biofilms. In general, multispecies biofilms were more resistant to inactivation and removal than single biofilms. Multispecies biofilms with all the six bacteria had the highest resistance to SHC, while those without *A. calcoaceticus* were the most susceptible. Only biofilms without *A. calcoaceticus* were not able to recover their biomass from the SHC treatments. *A. calcoaceticus* has a key role in the resistance and functional resilience of DW biofilms formed by the tested bacteria (the single species biofilms formed by this bacterium were from the most susceptible to SHC; however, its presence in multispecies biofilms increased their resistance to disinfection and their ability to recover from SHC exposure).

A. calcoaceticus, B. cepacia, Methylobacterium sp. and *M. mucogenicum* single species biofilms were strongly inhibited by the cell-free supernatants from the other bacteria. *M. mucogenicum* and *Sph. capsulata* cell-free supernatants demonstrated a high potential to inhibit the growth of counterpart biofilms. For multispecies only cell-free supernatants produced by *B. cepacia* and *A. calcoaceticus* had no inhibitory effects (caused potentiation) on multispecies biofilm formation. Multispecies biofilms were highly susceptible to metabolite molecules in the absence of *A. calcoaceticus*.

These conclusions clearly reveal that *A. calcoaceticus* had the highest ability to adhere to surfaces, coaggregated with partner bacteria and provided resistance and resilience to control conditions. The presence of this bacterium in the tested synthetic consortium represented a significant colonization advantage. It seems strategic to consider the presence

of this bacterium in the local DW system as a predictor of the presence of SHC resistant biofilms. Also, species association increased biofilm resistance and resilience to control conditions comparatively to single species biofilms.

10.2 Perspectives for further research

Much more needs to be studied on the mechanisms of biofilm formation in DWDS, on the role of each colonizing species in biofilm formation and resistance and the impact of each biofilm former species to the public health.

The characterization of microbial interactions between bacteria and other microorganisms, including fungi and protozoa will provide a more detailed picture on the complex interactions established in a real DW biofilm.

The study of intergeneric species interactions, adhesion and coaggregation at the nano-scale (using atomic force microscopy) will provide new and relevant information, contributing to the knowledge of the early stage mechanisms involved in biofilm formation.

It seems fundamental to understand the role of each biofilm colonizers in the public health. In this context, tests of infection with a defined human cell line will provide new insights on role of waterborne pathogenesis, mainly those opportunistic on immunocompromised individuals. Also, tests on antibiotic resistance could provide interesting information on the public health impact of infections caused by those bacteria.

The detailed characterization (by chromatography and nucleic magnetic resonance) of the metabolite molecules will provide information on their chemical nature, concentrations and novelty. If a new and relevant molecule will be identified, studies on the optimization of production should be conducted.

The detailed characterization of biofilm resistance mechanisms due to species association could provide relevant information that can contribute to a more efficient

biofilm control. It is clear that biofilms are more resistant to antimicrobials than their planktonic counterparts. This is mainly due to mass transfer limitations and chemical interaction between EPS and the antimicrobial. However, other and so far unknown mechanisms can be behind the increased resistance of multispecies biofilms.

The role of *A. calcoaceticus* on biofilm formation and resistance demonstrated in this thesis proposes that additional physiological characterization on its surface properties can help to explain such properties. This characterization could be focused on the presence of extracellular appendages, production of extracellular metabolites and on the characteristics of the outer membrane, mainly those proteomic.

The study developed for this thesis demonstrated that the most commonly used antimicrobial chemical (SHC) to control the microbiology of DWDS was inefficient in the control of biofilms formed by DW-isolated bacteria. It seems clear that new control strategies need to be investigated and implemented. The use of new antimicrobial chemicals with high ability to penetrate through the biofilm layers, with low interaction with EPS and low cytotoxicity will contribute to a more efficient biofilm control with potential application in DW disinfection. Even knowing that SHC is inefficient in biofilm control, their removal from DWDS disinfection protocols is not recommended. However, its synergistic association with new antimicrobials can contribute to the increase of DW quality.