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FEUP

Biofilm Dynamics and Drinking Water Stability:

Effects of Hydrodynamics and Surface Materials

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“Slime city - Chic, urban, sophisticated and sometimes deadly -
such are the inhabitants of the world's weirdest metropolis...

...Inside, shielded from harm and replete with every creature comfort, live
beings whose cosmopolitan lifestyle is only now coming to light. Amazing
cityscapes like this have existed on Earth for billions of years, built and
populated by plain, humble bacteria such as...”

(Coghlan, *New Scientist*, 1996)

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Publications

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Abstract

Biofilms are a major problem in drinking water systems. They contain a very high percentage of microorganisms which are protected against disinfectants attack and their life conditions are improved. A good knowledge of biofilm growth and detachment under conditions similar to those occurring in real systems may help on water systems design and on preventing risky events for public health.

In this work bacteria accumulation, both in suspension and attached to surfaces, was followed under steady (turbulent, laminar and no-flow) and unsteady conditions (intermittent flow) in laboratory reactors fed with drinking water. This water was practically neutral with low contents of dissolved solids, carbon, phosphorous, nitrogen and chlorine. Around $2 - 5 \times 10^5$ total cells were present per milliliter of water in which 22 - 45 % were metabolically active and 0.05 - 1.5 % cultivable. Drinking water contained *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* with predominance to the *Betaproteobacteria* (32 - 58 %). A low number of nitrifying bacteria was also found in the drinking water. A high fraction of cultivable bacteria (90.3 %) was composed of negative rods, from which 67.6 % were identified to genera *Pseudomonas*, *Xanthomonas*, *Flavobacterium*, *Stenotrophomonas* and *Sphingomonas*. The opportunistic pathogen *Pseudomonas aeruginosa* was not detected in any of the analyzed water samples.

Hydrodynamic conditions are of great importance on the behavior of the bacteria in the biofilm as they affect their accumulation and detachment. Under steady flow conditions, biofilms were composed of $0.1 - 5 \times 10^7$ total cells/cm², 17 - 34 % of them being metabolically active and 6 - 18 % cultivable. The growth rate of bacteria attached to PVC was 0.15 day⁻¹ while the growth rate for bacteria in suspension, was similar to the inverse of the residence time, *i. e.* 1.8 day⁻¹. Under steady state conditions, a higher water velocity caused a decrease in the biofilm growth rate, as well as a decrease in the cells wall accumulation due to a higher detachment rate. The residence time, the stagnancy and temperature enhanced cell accumulation in biofilms and in the water phase, as well as their metabolic activity and cultivability.

Unsteady flow conditions contributed to the formation of stable biofilms with decreasing fractions of metabolic activity and cultivability with biofilm depth and life length. Flushing systems subjected to stagnant periods promoted a release of a very high amount of biofilm cells, thus degrading the water quality.

Several materials were tested for their biofilm growth potential. The roughness and the hydrophobic character of the polymeric materials (PVC, PEX, HDPE and PP) contributed to an increasing biofilm accumulation in flow systems. These effects were negligible under stagnant conditions. The effect of hydrophobicity was not clear for

stainless steel pipes with and without surface modifications (SiO_x, Excalibur, DLC and Ni-P-PTFE coatings).

Microbial populations in biofilms were affected by the hydrodynamic conditions and the surface materials, although *Betaproteobacteria* was always the dominant group. For instance, biofilms formed in turbulent flows were much more cohesive and less prone to detach than those formed under laminar flow or stagnant conditions and, thus their composition had a slower variation over time.

Keywords: drinking water, biofilm, microbial population, kinetics, adhesion material, roughness, hydrophobicity.

Résumé

Les biofilms représentent un problème majeure lié à la présence de grand nombre de micro-organismes protégés contre les attaques des désinfectants et aussi à un environnement favorable. Une meilleure connaissance de la croissance du biofilme et de son détachement dans des conditions similaires à celles des systèmes réels peut contribuer à la création des systèmes d'eau et permettre la prévention des risques qui peuvent affecter la santé publique.

Dans ce travail l'accumulation des bactéries, en suspension et attachées à des surfaces, a été étudié sous des conditions dynamiques (instable, laminaire et sans flux) dans un réacteur de laboratoire alimenté avec de l'eau potable. L'eau potable était pratiquement neutre avec un faible contenu en solides dissolus, carbone, phosphate, nitrogène et chlore. Sommairement, $2 - 5 \times 10^5$ cellules par millilitre d'eau étaient présentes dont 22 - 45 % étaient métaboliquement actives et 0.05 - 1.5 % cultivables. L'eau potable a contenu *Proteobacteria*, *Bacteroidetes* et de *Actinobacteria* avec une prédominance de la sous-classe *Betaproteobacteria* (32 - 58 %). Un faible nombre de bactéries nitrifiées était présent dans l'eau potable. 90.3 % des bactéries cultivables étaient allongées et Gram-négatifs, auxquelles 67.6 % étaient identifiées comme *Pseudomonas*, *Xanthomonas*, *Flavobacterium*, *Stenotrophomonas* et *Sphingomonas*. Le microbe pathogène opportuniste *Pseudomonas aeruginosa* n'a pas été détecté dans aucun des échantillons analysés.

Les hydrodynamiques jouent un rôle important dans le comportement des bactéries dans les biofilms. Ils influencent l'accumulation et le détachement. Dans les conditions stables, les biofilms étaient composés de $0.1 - 5 \times 10^7$ cellules totales /cm² avec 17 - 34 % métaboliquement actives et 6 - 18 % cultivables. Le taux de croissance des bactéries attachées au PVC était de 0.15 jour⁻¹. En suspension, ce taux était similaire à l'inverse du temps de résidence (1.8 jour⁻¹). La vitesse de l'eau a diminué la croissance du biofilme ainsi que son accumulation aux parois, dans des conditions stables, due à un niveau élevé de détachement. Le temps de résidence, la stagnation et la température ont augmenté l'accumulation des cellules dans les biofilms et dans la phase d'eau ainsi que leur activités métaboliques et cultivabilités.

Les conditions de flux instables ont contribué à la formation de biofilms stables avec une diminution d'activité métabolique et de culture proportionnelle à la profondeur du film et au temps. Les systèmes de flux en périodes stagnantes favorisent le relargage d'un niveau élevé de cellules de biofilms et par conséquent corrompent la qualité d'eau.

Plusieurs matériaux ont été testés relativement à leur potentiel de croissance de biofilme. La rugosité et le caractère hydrophobique augmentent l'accumulation de biofilms dans les systèmes de flux quand il s'agit des matériaux polymériques (PVC,

PEX, HDPE et PP). Ces effets étaient négligeables dans les conditions stagnantes. Cependant, l'effet d'hydrophobicité n'était pas clair dans le cas du acier inoxydable, avec ou sans modifications de surface (SiOx, Excalibur, DLC et Ni-P-PTFE).

Les populations microbiennes des biofilmes ont été affectées par les conditions hydrodynamiques et de la surface du matériau. Par exemple, les biofilmes formés dans un flux turbulent étaient plus cohésifs et moins susceptible au détachement permettant ainsi une réduction de variabilité avec le temps.

Mots-clés: eau potable, biofilme, population microbienne, cinétique, matériel d'adhérence, rugosité, hydrophobicité.

Resumo

Os biofilmes representam um dos maiores problemas em sistemas de água potável, contendo uma elevada fracção de microrganismos que se encontram protegidos dos desinfectantes e as suas condições de vida são melhoradas. Um bom conhecimento do comportamento dos biofilmes em condições semelhantes às reais pode ajudar na construção destes sistemas e na prevenção de eventos com risco para a saúde pública.

Neste trabalho, a acumulação de bactérias, suspendidas ou aderidas às superfícies, foi seguida em condições estacionárias (turbulento, laminar e estático) e não estacionárias (escoamento intermitente) em reactores laboratoriais alimentados com água da torneira. A água potável é praticamente neutra com baixos conteúdos em sólidos dissolvidos, carbono, fósforo, azoto e cloro. Cerca de $2 - 5 \times 10^5$ células totais estão presentes em cada mililitro de água das quais 22 - 45 % são metabolicamente activas e 0,05 - 1,5 % cultiváveis. A população microbiana da água potável é composta por *Proteobacteria*, *Bacteroidetes* e *Actinobacteria* com predominância da subclasse *Beta* das *Proteobacteria* (32 - 58 %). Baixos teores de bactérias nitrificantes também foram encontrados. Uma fracção elevada das bactérias cultiváveis (90.3 %) era composta bastonetes Gram-negativos dos quais 67.6 % foram identificados como *Pseudomonas*, *Xanthomonas*, *Flavobacterium*, *Stenotrophomonas* e *Sphingomonas*. A bactéria patogénica oportunista *Pseudomonas aeruginosa* não foi detectada em nenhuma amostra.

A hidrodinâmica afecta muito a acumulação e o desprendimento das bactérias nos biofilmes. Em condições estacionárias de fluxo, os biofilms são compostos por $0,1 - 5 \times 10^7$ células/cm², sendo 17 - 34 % metabolicamente activas e 6 - 18 % cultiváveis. A taxa de crescimento das bactérias aderidas ao PVC foi 0,15 dia⁻¹ e, em suspensão, a taxa foi semelhante ao inverso do tempo de residência (1,8 dia⁻¹). Em condições de fluxo estacionárias, a velocidade da água diminui a taxa de crescimento do biofilme assim como a sua acumulação devido ao aumento do desprendimento. O tempo de residência, a estagnação e a temperatura aumentam a acumulação de células aderidas e plasmónicas, assim como a sua actividade metabólica e a cultivabilidade.

As condições não estacionárias de escoamento contribuíram para a formação de biofilmes estáveis com decrescentes fracções de actividade metabólica e de cultivabilidade com a espessura e a idade do biofilme. A imposição de elevados caudais de escoamento, após períodos de estagnação, promoveu a libertação de quantidades muito significativas do biofilme, degradando a qualidade da água.

O potencial de crescimento do biofilme foi testado para vários materiais. O aumento da rugosidade e do carácter hidrofóbico aumentaram a acumulação de biofilmes em sistemas de fluxo para os materiais poliméricos (PVC, PEX, HDPE e PP). Estes efeitos foram reduzidos em condições estacionárias. O efeito da hidrofobicidade não foi claro

para o aço inoxidável com ou sem modificações de superfície (revestimentos de SiO_x, Excalibur, DLC e Ni-P-PTFE).

As populações microbianas dos biofilmes foram afectadas pelas condições hidrodinâmicas e pelo material de adesão, embora *Betaproteobacteria* seja sempre o grupo dominante. Por exemplo, os biofilmes formados em escoamento turbulento eram muito mais coesos e com maior resistência ao desprendimento e, por isso verificou-se uma menor variabilidade da composição microbiana.

Palavras-chave: água potável, biofilme, população microbiana, cinética, materiais de adesão, rugosidade, hidrofobicidade.

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xxx

List of Abbreviations and Symbols

Abbreviations	Definition	Units
A	Internal surface area	cm ²
A _{biof}	Area of a biofilm coupon	cm ²
A _{field}	Area of the microscopic viewing field	cm ²
A _{memb}	Area of the membrane	cm ²
AOB	Ammonia oxidizing bacteria	
CTC	5-cyano-2,3-ditolyl tetrazolium chloride	
Cy3	5,5'-disulfo-1,1'-di(γ -carbopentynyl)-3,3,3',3'-tetramethylindolocarbo-cyanine (cyanine Cy3.18)	
D	Diameter	cm
DAPI	4',6-diamidino-2-phenylindole	
DGGE	Denaturing gel gradient electrophoresis	
DMSO	Dimethylsulfoxide	
DNA	Deoxyribonucleic acid	
DOC	Dissolved organic carbon	mg C/L
DVC	Direct viable counts	
d	Dilution factor	
dH ₂ O	Deionized water	
dNTPs	Mixture of deoxynucleotides	
EDIC	Episcopic differential interference contrast	
EDTA	Ethylene diamine tetraacetic acid	
EPS	Extracellular polymeric substances	
FA	Formamide percentage present during DNA hybridization	%
FISH	Fluorescent <i>in situ</i> hybridization	
FLUOS	5(6)-carboxyfluorescein- <i>N</i> -hydroxysuccinimide-ester	
HDPE	High density polyethylene	
HPC	Heterotrophic plate count	
HRT	Hydraulic residence time	
h	Reactor height	cm
IC	Inorganic carbon	mg C/L
NOB	Nitrite oxidizing bacteria	
n.d.	Not detected	
PAC	Probe active counts	
PBS	Phosphate buffered saline	mM
PC	Principal component	
PCA	Principal component analysis	
PCR	Polymerase chain reaction	

PEX	Cross-linked polyethylene	
PMMA	Polymethylmethacrylate	
PP	Polypropylene	
PTFE	polytetrafluoroethylene	
PVC	Polyvinyl chloride	
Q	Flow rate of recirculation	mL/h
Q _{ext}	Inlet flow rate	mL/h
R ₂ A	R ₂ A nutrient agar medium	
R _a	Arithmetic average roughness	μm
R _{max}	Maximum roughness depth	μm
RNA	Ribonucleic acid	
RQ	Non linear correlation coefficient	
R _q	Geometric average roughness	μm
R _z	Mean roughness depth	μm
r	Correlation coefficient	
rRNA	Ribosomal ribonucleic acid	
SEM	Scanning electron microscopy	
SDS	Sodium dodecyl sulfate	
s	Standard deviation	
t	Time	day, h
T	Temperature	°C
TAE	Tris (hydroxymethyl)aminomethano - Acetic - EDTA buffer	
TE	Tris-EDTA buffer	
TC	Total carbon	mg C/L
TDS	Total dissolved solids	
TEMED	Tetramethylethylenediamine	
TOC	Total organic carbon	mg C/L
u	Internal velocity	m/s
UPF	Ultra pure filtrated and 2x sterilized water	
V	Volume of the system	mL
VBNC	Viable but not cultivable	
V _{filt}	Filtered volume	mL
V _{susp}	Total suspension volume	mL
$\bar{X}$	Average of counted cell number	cell
X	Reactor suspended cell density	cell/mL
X _b	Biofilm cell number per unit surface area	cell/cm ²
(X _b) _{max}	Steady-state biofilm cell number per unit surface area	cell/cm ²
X _{in}	Inlet suspended cell density	cell/mL
X*	Steady state bulk suspended cell density	cell/mL
wt.	Weight/volume	

<i>Greek symbols</i>	Definition	Units
ΔG^{LW}	Apolar component of total free energy of hydrophobicity of a material surface	mJ.m^{-2}
ΔG^{AB}	Polar component of total free energy of hydrophobicity of a material surface	mJ.m^{-2}
ΔG^{TOT}	Total free energy of hydrophobicity of a material surface	mJ.m^{-2}
Φ_{att}	Rate of cell attachment to the surface	cells/h
Φ_{det}	Biofilm detachment or removal rate	cells/h
Φ_p	Biomass production rate in the biofilm	cells/h
β^{-1}	Average residence time of cells within the biofilm	day^{-1}
γ_i^{AB}	Acid-base component of surface free energy tension [i = S(surface); i = L(probe liquid)]	mJ.m^{-2}
γ_i^{LW}	Lifshitz–van der Waals component of surface free energy tension [i = S(surface); i = L(probe liquid)]	mJ.m^{-2}
γ_i^{+}	Electron-acceptor component of surface free energy tension [i = S(surface); i = L(probe liquid)]	mJ.m^{-2}
γ_i^{-}	Electron-donor component of surface free energy tension [i = S(surface); i = L(probe liquid)]	mJ.m^{-2}
γ_i^{TOT}	Total surface free energy tension [i = S(surface); i = L(probe liquid)]	mJ.m^{-2}
$\mu_{biofilm}$	Specific cell growth rate inside the biofilm	day^{-1}
$\mu_{biofilm}^{*}$	Steady-state specific biofilm cell growth rate in the biofilm	day^{-1}
μ_{bulk}	Specific cell growth rate in suspension	day^{-1}
$\mu_{overall}$	Overall specific cell growth rate	day^{-1}
θ_i	Contact angle i=B(α -bromonaphthalene); i=F(formamide); i=W(water)	$^{\circ}$
ω	Angular velocity	rpm

1. Introduction

1.1 Relevance and Motivation

Safe drinking water is essential for human health. All over the world, governments and water supplying companies are working on the improvement of drinking water quality, through the monitoring of its physicochemical and biological quality parameters together with the improvement of the design and the operational management of the distribution system.

Although the drinking water is closely monitored in the developed countries, waterborne outbreaks are still being reported in those countries due to pathogens contamination. The research community believes that biofilms contribute notoriously to these events by creating a protective and nutritive reservoir for pathogens growth or survival. However, it is not feasible to eradicate those biofilms from the drinking water distribution systems. Thus, the main goals concerning the drinking water biological quality are to control the extension of bacterial growth, to avoid the presence and/or growth of pathogenic organisms and to prevent any event that might have a hazardous effect in human health. This may only be achieved by seeking an integrated approach that covers a good choice of pipe materials, hydraulic conditions, and a close monitoring of the physical, chemical and microbiological parameters of the drinking water and of the attached deposits. Thus, it is necessary to have a good knowledge of biofilm accumulation and detachment,

under the operating conditions observed in drinking water distribution systems, including the effect of the surface materials.

Although the technologies and the monitoring have been improved, there are still several health risky events occurring nowadays due to emergent pathogens (bacteria, virus and protozoa). Other problems associated with the biofilm growth include microbially induced corrosion and unpleasant taste and odor.

Portuguese consumers seem to be still unsatisfied and suspicious about the drinking water quality, as published in the national newspaper *Jornal de Notícias* on 31st of May 2007. Accordingly to the report of the Instituto Regulador de Águas e Resíduos (national authority for water and waste management), almost half of the Portuguese population (45.3 %) are suspicious about the drinking water quality and prefer to ingest bottled water, and only 37.6 % drinks water from the public distribution system. Also, surveys of municipal water consumption in Canada and in U.S.A. (Jones *et al.*, 2007a) indicated a general negative perception of the municipal drinking water quality, and consequently, a high frequency of alternative water use is observed including bottled water and home water treatment devices.

1.2 Objectives and Outlines

The work presented in this thesis deals with bacteria accumulation, either suspended or attached, in reactor systems filled with local drinking water and where the operating conditions observed in real networks are simulated.

For this reason, biofilm and planktonic bacteria accumulation were monitored under steady state flow, stagnant water and intermittent hydraulic regimes. The materials tested for the biofilm growth potential were polymeric compounds, stainless steel and some new surface modified stainless steel materials.

The aim of this work was to find for the conditions that minimize both the presence of bacteria in reactors and their detachment from the surfaces in order to improve the drinking water quality.

The thesis is organized as follows. First, in Chapter 1, the relevance and motivation of the work are described as well as its main objectives. Then, the characteristics of drinking water systems, the biofilm growth and the factors that affect its accumulation in distribution networks, are presented. Chapter 2, Materials and Methods, describes the biofilm monitoring reactors and the analytical methods. Chapter 3, Drinking Water in the Distribution System, is a compilation of the

results obtained in terms of the physicochemical and microbiological parameters of the water coming directly from the tap. In Chapter 4, biofilm growth was monitored under continuous steady-state flow (laminar or turbulent) and in stagnant water. Kinetic parameters were quantified and discussed. Chapter 5 deals with biofilm accumulation under alternating flow conditions simulating daily tap usage. Chapter 6 concerns the effect of different materials and of their surface properties on the biofilm growth potential. An analysis of the surface properties is included. Finally, in Chapter 7 conclusions and suggestions for future work are presented.

1.3 Drinking Water and World Public Health

By the end of 2004, the General Assembly of the United Nations recognized that water is a critical point for a sustainable environmental development, an indispensable factor for human health and well-being and observed that half of population in developing countries still lives with scarcity of good quality water (Figure 1.1).

In 2005, a systematic review concluded that diarrhoeal episodes are reduced by 25 % through improving water supply, and by 39 % via household water treatment and safe storage (Fewtrell *et al.*, 2005). A more recent review of randomized controlled trials confirmed the key role that point-of-use water quality interventions could play in reducing diarrhea episodes, reporting a reduction in diarrhoeal disease morbidity by roughly half, on average, with some studies resulting in disease reductions of 70 % or more (Clasen *et al.*, 2006).

Several health risk events were reported about the diseases spread through unsafe water that acts as a passive carrier for the infecting pathogens (waterborne outbreaks). These pathogens were mainly bacteria, virus or protozoa and are listed in Table 1.1 (Ashbolt, 2004; WHO, 2006; Karanis *et al.*, 2007).

Generally, the origin sources are human and animal faeces and may persist or even multiply in water supplies for at least 1 week. Although the 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*.

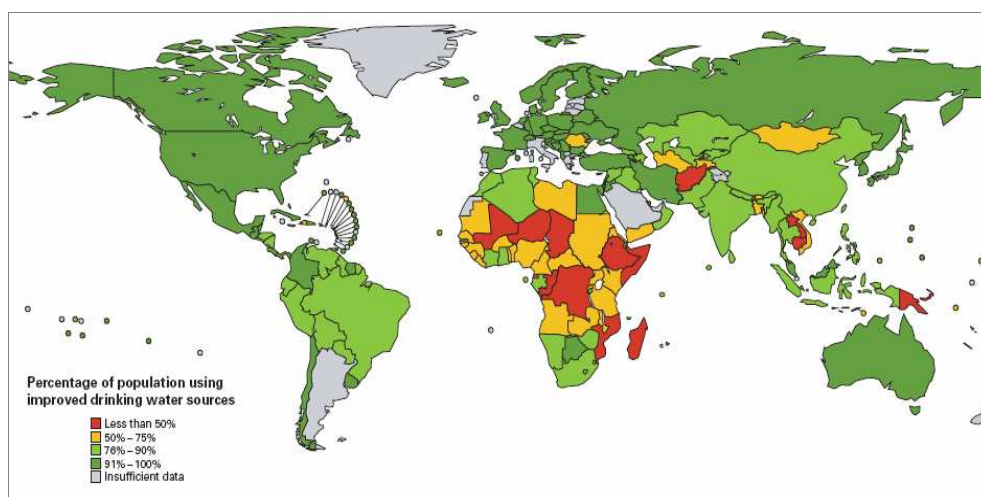


Figure 1.1 - Percentage of population using improved drinking water sources in 2004.
Source: WHO/UNICEF Joint Monitoring Programme for Water and Sanitation.

Table 1.1 - Pathogens associated to waterborne outbreaks due to drinking water ingestion.

Bacteria	Virus	Protozoa
<i>Pseudomonas aeruginosa</i>	Rotavirus	<i>Balantidium coli</i>
<i>Legionella pneumophila</i>	Calicivirus	<i>Giardia lamblia</i>
<i>Aeromonas</i> spp.	Norovirus	<i>Cryptosporidium parvum</i>
<i>Mycobacterium</i> spp.	Astrovirus	<i>Entamoeba histolytica</i>
<i>Campylobacter jejuni</i>	Adenovirus	<i>Acanthamoeba castellanii</i>
<i>Escherichia coli</i>	Hepatitis virus	<i>Naegleria fowleri</i>
<i>Yersinia enterocolitica</i>	Enterovirus	<i>Cyclospora cayentanensis</i>
<i>Helicobacter pylori</i>	Coxsackie virus	<i>Toxoplasma gondii</i>
<i>Salmonella</i> spp.	Echovirus	
<i>Shigella</i> spp.	Poliovirus	
<i>Vibrio cholera</i>		
<i>Leptospira</i> spp.		
<i>Burkholderia pseudomallei</i>		
Toxic cyanobacteria		

In 1995, the World Health Organization underestimated that 2.1 billion people were suffering from several water-related diseases, of which 14 million were deadly cases. In 2002, 17 % (1.1 billion) of the world population had no access to safe water and still 1.7 million was dying from waterborne diseases. Clearly, the older,

the younger and the immune-compromised people are the more affected groups due to the fact that these infections are dependent on the level of contamination and on the host susceptibility and immunity system response. In Figure 1.2, the fraction of the loss of healthy lives is presented and the values are much lower in the developed countries than in the other ones, due to a closed monitoring and control of the biological and chemical composition of drinking water.

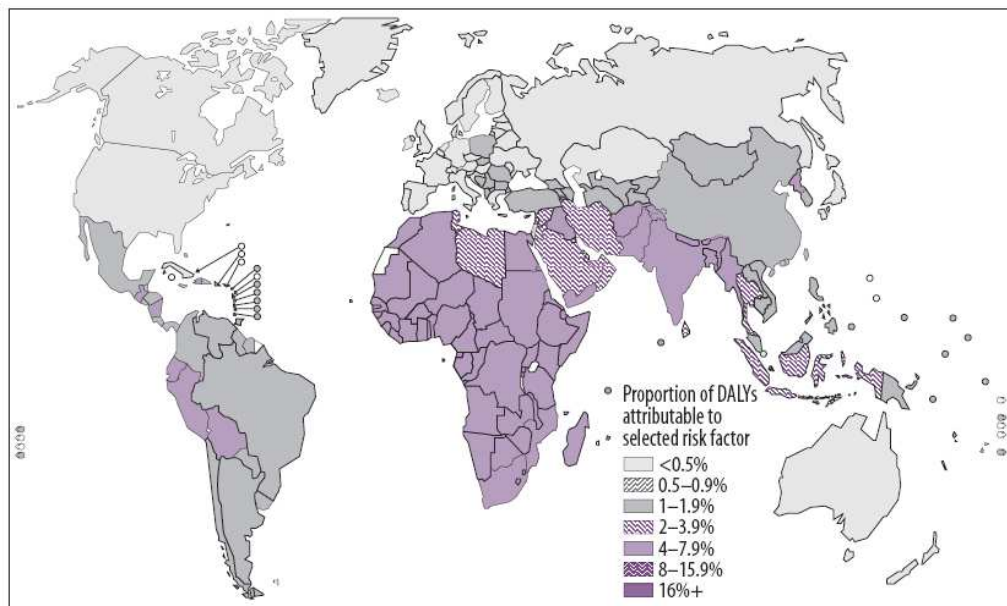


Figure 1.2 - Burden of disease attributable to unsafe water. The scale is in %DALY in each region. DALY unit (Disability-Adjusted Life Year) is equal to the loss of one healthy life year. The figure was presented in the 2002 WHO report.

Payment *et al.* (1991) suggested that approximately 35 % of the gastrointestinal infections were associated with the consumption of drinking water, in which 14 - 40 % of illnesses were attributable to tap water meeting current standards, as observed some years later (Payment *et al.*, 1997). The costs associated to these health effects could reach several millions dollars each year for Canada with a population of 30 million people.

Although the referred water-related diseases are seldom deadly in the developed countries, there are several authors reporting that drinking water 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 drinking water 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 *Escherichia coli* O157:H7, *Campylobacter* spp., *Helicobacter pylori*, *Pseudomonas 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; Amvrosyeva *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. And 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 U.S.A. These outbreaks were attributed to microbial contaminations with virus, parasites and pathogenic bacteria or to chemical/toxin poisonings.

Thus the conclusion seems obvious: attached or suspended microorganisms are not well controlled by water-related companies. Indeed, Payment (1999) observed that the disinfectant residual in drinking water did not provide a significant inactivation of pathogens and possibly masks events of contamination in the distribution. Moreover, protozoan parasites and virus were observed to persist within drinking water biofilms and, thus, being released to the water phase for several days after inoculation, under either constant laminar or intermittent flow (Helmi *et al.*, 2008). Bacterial pathogens were observed as being well incorporated in biofilms that acts as a safe harbor against the disinfectant residual (Altman *et al.*, 2007).

Chemical water quality seems generally of lower importance as the impact on health tends to have chronic long term effects and time is available to take remedial actions. Several researchers (Ning, 2002; Ramsay and Hankin, 2005; Ward *et al.*, 2005) emphasized that acute effects may only be found where a major pollution event has occurred or where levels of certain chemicals are high in the raw natural sources such as fluoride and arsenic, or anthropogenic sources, such as nitrate. Actually, in the United Kingdom, over a quarter of the drinking water tested breach the European Union legislation for nitrate, nitrite, lead, copper and sulfate levels (Harrison *et al.*, 2000).

1.4 Drinking Water Distribution System

The chemistry and composition of drinking water vary enormously in different distribution systems spread all over the world. It depends on the type of raw water (*e.g.* ground water or river water) used in the treatment plant, the processes applied to purify the water and on the characteristics of the distribution network (*e. g.*, temperature, disinfectant residual, nutrients, flow velocity profile and pipe materials). Generally, these systems are oligotrophic and neutral environments with low contents of dissolved or suspended substances, which will limit *per se* microbial growth.

Many drinking water distribution networks are designed for target liquid velocities of 0.2 - 0.5 m/s that may correspond to laminar or turbulent flow. In most fixed biomass systems, stable and higher flow rates have the advantage of limiting biofilm growth (Peyton and Characklis, 1993; Melo and Vieira, 1999; Cloete *et al.*, 2003) since under this turbulent regime they produce thinner and more cohesive layers less prone to release bacteria into the bulk water. However, these conditions are not always feasible to maintain in drinking water networks. The hydraulic conditions in drinking water systems range from laminar to turbulent flow, but stagnant (non-flow) waters also occur in places where the water consumption is low, as well as in reservoirs in buildings (Ayoub and Malaeb, 2006).

In a ***drinking water treatment*** plant, the objective is to obtain drinking water that fulfills the requirements of consumers and the authorities defined by several organoleptic, microbiological and physicochemical parameters. Generally, the process is composed by filtration, coagulation and disinfection. Figure 1.3 shows a scheme of a water treatment plant where the raw water is coming from a river.

Initially, the river water is captured to a storage tank and pre-disinfected to minimize the growth of microorganisms and, consequently, the biofouling in the pipes and tanks. If the water is acidic then pH is increased to low basic values by adding lime or soda ash, which is very important for the flocculation process efficiency.

Filtration is generally composed of different units depending on the type of the substances to be removed, on the size of particles in suspension and on the water purification degree. Filtration may be performed simultaneously with the ***flocculation*** in order to increase the particles removal efficiency. The aim of

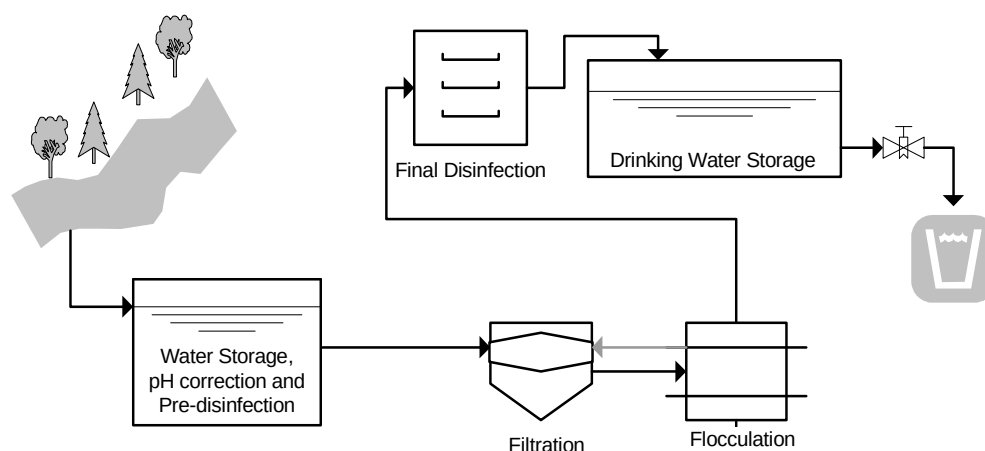


Figure 1.3 - Scheme of a treatment plant and distribution system for drinking water.

flocculation is to produce particles or flocs of bigger size that can be removed by settlement, flotation or filtration. The efficiency of flocculation is dependent on pH, the flocculants and on the water composition. The most used flocculants are metals salts, such as iron and aluminum salts (Bagga *et al.*, 2008).

In the simultaneous process of filtration/flocculation, raw water firstly passes through a granular bed of sand with anthracite to remove most of the particles in suspension, especially if a coagulant was added previously. Secondly, the filtration through a media rich in calcium carbonate will retain almost all the particles and the pH is neutralized. This step will have higher efficiency of particles removal with a pre-addition of a polyelectrolyte that will promote the agglomeration of particles in suspension. Finally, water is filtered through a layer of activated carbon to remove the excess of contaminants. At the end of this process the water is clear, colorless and tasteless.

Final **Disinfection** of water is used to eliminate the microorganisms, pathogenic or not, that have passed through the treatment processes and to guarantee microbiologically safe water through the distribution system to the point of use. This is achieved by adding disinfectant in excess, which will maintain a residual concentration during the water distribution process 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).

Chlorine is the disinfectant most commonly used in drinking water. Chlorination is performed with sodium hypochlorite that will release Cl_2 into the aqueous phase by hydrolysis. One drawback is that free chlorine reacts with natural organic

compounds and form potentially harmful by-products (such as trihalomethanes, haloacetonitriles and haloacetic acids) (Bull, 1982; Speitel *et al.*, 2005; Smith, 2007). These compounds were identified as potentially harmful (carcinogenic or mutagenic) for human health (Bull, 1982; Smith, 2007). Furthermore, this chemical disinfection may affect taste and odor. Although very effective in killing bacteria, free chlorine has a moderate effect against virus and a very limited effect against protozoa (WHO, 2006).

In the presence of ammonia, hypochlorite reacts forming chloramines that are weaker disinfectants. These chemicals are widely applied in drinking water distribution system (chloramination) because produces the same harmful by-products as chlorine in lower amounts (Speitel *et al.*, 2005). Their residual concentration is kept for longer periods in such systems and chloramines are not as reactive as chlorine with the iron and corrosion products in pipe matrixes (LeChevallier *et al.*, 1990). Water distribution systems disinfected with chloramines may promote the occurrence of nitrification, wherein ammonia is used a nutrient for bacterial growth, with nitrates being generated as a by-product. Nowadays researchers proposed the combined use of chlorine and monochloramine in order to reduce the by-products contamination (Momba and Binda, 2002; Charrois and Hrudey, 2007; Chowdhury *et al.*, 2007).

Chlorine dioxide is another effective water disinfectant, not widely used, that does not produce trihalomethanes nor react with ammonia. When applied in low amounts concomitantly with chlorine the formation of trihalomethanes decreased significantly (Lee and Nam, 2005). Some concern has been raised over possible effects of its degradation by-products (chlorite or chlorate).

Ozonation has been shown to remove efficiently microorganisms, taste and odor. 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 dangerous by-products, as compared with chlorine, and leaves no taste or odor. The use of ozone should be avoided with waters with bromine as both react and generate bromate, a suspected carcinogen. On the other hand, as a strong oxidizing agent, ozone can decrease total organic carbon, but it increases the assimilable organic carbon and microbiologically available phosphorous, which promote microbial growth in distribution systems (Zacheus *et al.*, 2000; Lehtola *et al.*, 2002). Unfortunately, its solubility in water is relatively low, which limit its application; especially in maintaining a residual amount during the water distribution.

Ultraviolet (UV) radiation is a very effective disinfectant against all bacteria, virus and protozoa, cysts included (Clancy *et al.*, 2004; Hijnen *et al.*, 2006) in clarified waters. The main disadvantage is that UV leaves no residual disinfectant in the water, which is overcome by applying a second disinfectant to generate a residual amount.

Other optional purification treatments may be applied depending on the characteristics on the raw water, such as, the removal of radium and lead. Curiously, the drinking water is fluoridated to improve tooth health in many countries, such as U.S.A., Australia, New Zealand, South Africa and Republic of Ireland.

However, van der Kooij and colleagues (1999a; 1999b; 2000) emphasized that biologically stable water can be distributed without any disinfection if there is effective removal of nutrients during the water treatment plant. Most European companies were using chlorine as a disinfectant in 1998 (Lenntech, 2008), such as Portugal. Alternative options were applied in France (mainly, ozone). Italy and Germany uses chlorine just for maintaining the disinfectant residual. Great Britain uses chloramines for residual disinfection in the distribution network. Finland, Spain and Sweden use chloramines for disinfection occasionally. And, in Denmark, no disinfection is performed.

In drinking water distribution systems, the density of suspended bacteria increases between the treatment plant and the consumer's tap as a function of the disinfectant decay, hydraulic residence time, substrate uptake and the presence of corrosion deposits. In a drinking water piping system with a volume/surface area ratio of 5 cm, 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 through detachment phenomena (van der Wende *et al.*, 1989), which depends on a variety of factors such as hydrodynamic patterns and surface materials.

1.5 Attached versus Planktonic Bacteria

Life of bacteria attached in biofilms offers many advantages. As observed by early researchers (Zobell and Anderson, 1936), bacterial growth in stored samples was truly improved as the surface-volume ratio increased. 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 from the biofilm. For example, in biofilms certain bacteria produce extracellular enzymes capable of hydrolyzing proteins, nucleic acids and polysaccharides, thereby generating soluble products that are metabolized by these organisms or by neighboring bacteria lacking this hydrolytic capability. Moreover, the fact that the attached bacteria were bigger than the planktonic ones may indicate that life was improved in the biofilm.

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). An example is the initial co-aggregation observed between two species after adding a nutrient metabolized just by one of them. If the nutrient was metabolized by both species, biofilm started growing with separate microcolonies of each specie (Nielsen *et al.*, 2000). Also, this improved spatial organization has been demonstrated for nitrifying and sulfate reducing biofilms (Schramm *et al.*, 1999). Biofilm bacteria communication through excreted signaling molecules (chemotaxis) is another major advantage of living in communities (Wadhams and Armitage, 2004; Wuertz *et al.*, 2004). Allesen-Holm *et al.* (2006) observed that biofilm with bacteria unable to produce signaling molecules (acyl homoserine lactone and quinolone) produced less extracellular DNA and were more susceptible to biocide attack.

1.6 Biofilm

Biofilms are formed ubiquitously in any interface (liquid or solid) in contact with water. Drinking water biofilms are composed by complex microbial communities functionally organized and embedded in a gelatinous matrix of extracellular polymers excreted by microorganisms. Any inorganic particles passing nearby (corrosion products, clays, sand ...) may also be incorporated in the biofilms 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 the low sizes, high growth rates, adaptation capacity and extracellular polymers production. This fact explains the reason why most of the

times, cells in biofilm are referred as bacteria, as happens also in this thesis. In addition, virus, protozoa, fungi and algae may be incorporated in drinking water biofilms as observed by several authors (Sladeckova, 1998; Gaylarde and Morton, 1999; Momba *et al.*, 2000; Codony *et al.*, 2003; Snelling *et al.*, 2006).

A mature biofilm may be formed within 5 days to 4 months in a drinking water system (Block *et al.*, 1993; Kerr *et al.*, 1999; Volk and LeChevallier, 1999; Zacheus *et al.*, 2001; Bachmann and Edyvean, 2006) reaching 10^7 - 10^9 cells per square centimeter of surface material with thicknesses ranging from 10 micron to few centimeters (Melo, 2003) as may be observed in Figure 1.4.

The major component of the biofilms is undoubtedly water, representing from 70 to 99 % (Watnick and Kolter, 2000; Flemming *et al.*, 2002; Stoodley *et al.*, 2002). Generally, biofilms are visco-elastic and colored white/yellow to brown/black. As EPS have a mucilaginous consistency, the adsorption of particles and cells may occur, even for those that are not able to produce adhesion polymers. If pathogens were present in the water phase, even in low concentrations, they may found within the biofilm favorable growth conditions. Consequently, their accumulation and detachment constitute a high risk to public health because pathogens will be released to the water at concentrations that represent an infectious dose.



Figure 1.4 - Pipe sections from a drinking water distribution system made of (a) - ductile iron with high amounts of corrosion products and biofilm in Chaves (2004) and (b) - cement cast iron with high accumulation of biofilm and inorganic particles.

The biofilm matrix (composed by all inorganic and organic substances surrounding the cells) has several functions. Besides 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, 2001b). Also 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 of metabolites (Wadhams and Armitage, 2004; Wuertz *et al.*, 2004).

Extracellular polymeric substances (EPS), the major component of biofilm matrix, are considered the organic substances excreted by the attached microorganisms and are important keys for the biofilm start-up (Watnick and Kolter, 2000; Melo, 2003). Studies with bacteria isolated from a wastewater treatment reactors concluded that the adhesion of cells was inhibited through electrostatic interaction when the EPS amount was low and enhanced through polymeric bridges when EPS was present at high quantity (Azeredo and Oliveira, 2000; Tsuneda *et al.*, 2003).

EPS are composed of proteins, humic substances, polysaccharides, lipids and ribonucleic acids (Frolund *et al.*, 1996; Azeredo *et al.*, 1999; Zhang *et al.*, 1999; Sutherland, 2001a). Their composition and amount are highly dependent on 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, *e.g.* nitrogen and phosphate, promoted exopolysaccharides synthesis (Sutherland, 2001b; Melo, 2003). River biofilms produced different amounts of polysaccharides when nutrients availability and flow velocity changed (Staudt *et al.*, 2004). Extracellular polysaccharides, proteins, lipids and sugars may be stained for viewing in a Laser Scanning Microscopy using specific lectins/dyes (Chen *et al.*, 2007). For example, Concanavalin A is specific for mannose sugars present in the EPS and cell wall of *Pseudomonas aeruginosa*; peanut lectin has high specificity for D-galactose that is present in *Burkholderia cepacia* EPS (Harrison *et al.*, 2006).

1.6.1 Biofilm Formation

Biofilm development on a solid surface in contact with water is schematically represented in Figure 1.5. The main processes involved in the biofilm build-up are:

- 1) formation of a conditioning film;
- 2) initial adhesion of microorganisms to the surface;
- 3) biofilm growth;
- 4) biofilm maturation - equilibrium between accumulation and detachment.

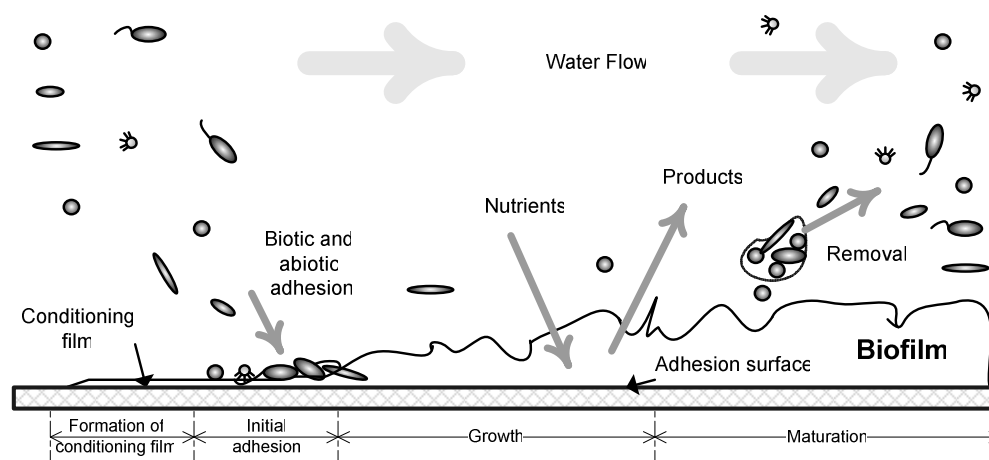


Figure 1.5 - Processes involved in the biofilm growth. Adapted from Melo (2003).

Conditioning Film. 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) and low adsorption energies ($\Delta H_{\text{ads}} \leq 50 \text{ kJ mole}^{-1}$). In chemical adsorption, several adsorbed molecular layers are formed and involves specific chemical bonds (electrostatic, covalent and hydrogen bonds), dipole interactions, and hydrophobic interactions. In this case, the adsorption energies are higher ($\Delta H_{\text{ads}} = 80 - 420 \text{ kJ mole}^{-1}$) (Marshall, 1996). The strength of the biofilm adhesion is largely dependent on the cohesion of the conditioning film as observed by several authors (Busscher and Van Der Mei, 1995; Bos *et al.*, 1999).

Initial Adhesion. Microorganisms are transported towards the 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 adhesion. As a result of superior transport properties, flagellated cells of *Pseudomonas fluorescens* colonized a substratum at a higher rate than unflagellated cells (Mueller, 1996).

The first arriving bacteria adhere to the surface initially through weak, reversible van der Waals forces. If not immediately separated from the surface, the bacteria are anchored more permanently by developing stronger bonds for the attachment to the surface (for example, by active cell biosynthesis of extra cellular polymers). Different mechanisms had been proposed to explain the bacteria attachment to

surfaces with different hydrophobicity character (Marshall, 1996). The non polar groups of fimbriae, lipopolysaccharide and outer membrane proteins may allow the bacterium to approach hydrophobic surfaces. On the other side, there are also polymers capable of hydrogen bonding or electrostatic interactions (*e.g.*, polysaccharides) that mediate the attachment to hydrophilic surfaces. Also important is the presence of other bacterial surface polymers that may prevent adhesion. An example is the attachment of *Pseudomonas* sp. to hydrophobic and hydrophilic surfaces through different mechanisms: on hydrophobic surfaces, the adhesion is quicker and more irreversible and the adhered amount was higher than on hydrophilic surfaces (Wiencek and Fletcher, 1995). In a different research work (Brizzolara and Holm, 2006), higher amount of *Pseudomonas fluorescens* biofilm was also obtained on most hydrophobic surfaces although with a lower cohesion strength.

Biofilm Growth. The attached bacteria start growing, excreting organic polymers, and initiating the formation of the biofilm matrix. Tolker-Nielsen *et al.* (2000) observed that the first micro-colonies formed were mono-species resultant of the growth of the attached bacteria and not of cell aggregation. Further attachment of planktonic bacteria and of inorganic particles will contribute to a structurally heterogeneous biofilm growth, as well as, migration of attached bacteria between and inside of micro-colonies (Klausen *et al.*, 2003). 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 the biofilm maintenance. During this phase, bacteria detachment events occur although at a lower extent compared to the growth rate.

Biofilm maturation. Mature biofilm are composed of an organized consortia of microorganisms embedded in an organic matrix that protects the bacteria. The structure of a mature biofilm depends on the microbial composition, EPS production, the nutrient availability, hydrodynamic conditions and temperature. In a biofilm several processes may occur simultaneously: bacteria detachment into water, attachment of planktonic bacteria, growth, death...

However in a mature biofilm these processes are at equilibrium and the attached cells per unit surface area are constant with time although with periodic fluctuations. 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.

A typical picture of biofilm accumulation with time is presented in Figure 1.6 in which an initial lag phase (bacteria adaptation to the medium) followed by biofilm growth until quasi-steady state is achieved. The stationary phase with negligible microbial growth is due to the densely settled area surrounded by exopolysaccharides or growth and death of microbes, and attachment and detachment are in balance.

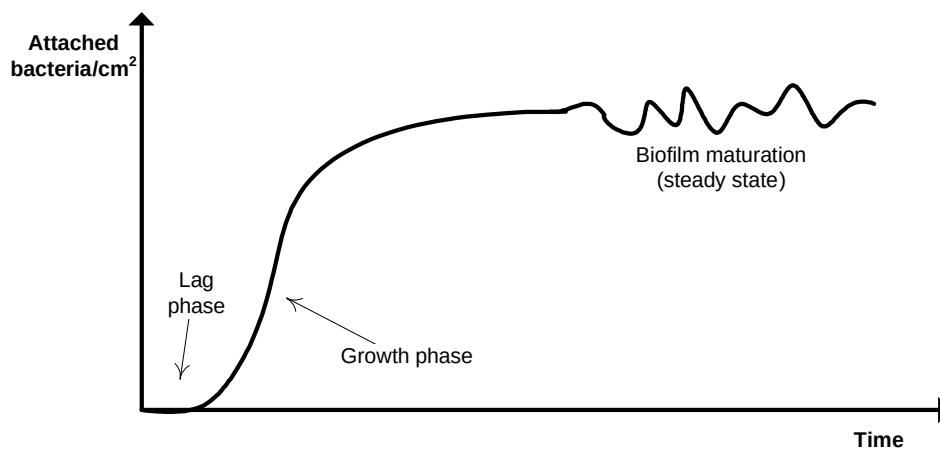


Figure 1.6 - Biofilm evolution with time. Adapted from Caldwell (1995).

Detachment. Several factors were observed to promote detachment of biofilm portions or of isolated bacteria and were attributed to perturbations in the environmental conditions and a consequent active response of biofilm bacteria. These factors are the following.

- a) The increase of shear stress, alternating flow conditions and abrasion due to particle collisions promote sloughing off of biofilm pieces that were not well cohesive (Duddridge et al., 1982; Powell and Slater, 1982).
- b) Starvation of bacteria has been demonstrated to promote size reduction, and increase of bacteria fragmentation and of motility (Mueller, 1996; Hunt *et al.*, 2004) which will increase biofilm detachment. Gjermansen *et al.* (2005) observed that these events were attributed to EPS dissolution by proteins excreted by starved *Pseudomonas putida*. This EPS dissolution was also observed in a *Pseudomonas aeruginosa* biofilm that excreted alginate lyase enzyme which cleaves the polymer into short oligosaccharides (Boyd and Chakrabarty, 1995).

- c) The increase of nutrients promoted the release of bacterial cells up to 80 % the total attached ones. This biofilm dispersion phenomenon was associated with increased expression of flagella genes (Sauer *et al.*, 2004).
- d) A chemical change in the EPS due to presence of chelating agents (Ca^{2+}) that will reduce the cohesive strength of the attached cells (Chen and Stewart, 2002).
- e) The excretion of surface-modified products (surfactants) by certain bacteria may promote detachment (Rosenberg and Ron, 1999).
- e) Bacteria detachment was observed to increase as the nutrients decrease inside the biofilm, which was attributed to high nutrient consumption and/or low mass transport.
- f) In biofilms the excretion of certain signaling molecules induced the detachment events (Wadhams and Armitage, 2004; Wuertz *et al.*, 2004).

1.6.2 Biofilm Structure

Several structures had been proposed as biofilm visualizing techniques were improved through the years. One of the first studies of freshwater biofilm observation with a microscopic technique reported "...a great variation in size, form, and arrangement of the cells, which, if observed systematically, must lead to some orderly understanding of the number and distribution of the species." (Henrici, 1933).

Some decades after, new microscopy technologies developed and biofilm was represented as a simple planar and compacted structure with a relatively constant thickness (Wimpenny *et al.*, 2000).

Meanwhile EDIC microscopy was developed by Keevil and Walker (1992) and the heterogeneous mosaic model was proposed for biofilms growing on the inner surfaces of drinking water distribution systems (Figure 1.7a). Micro-colonies of bacteria adhered by extracellular polymeric substances (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.

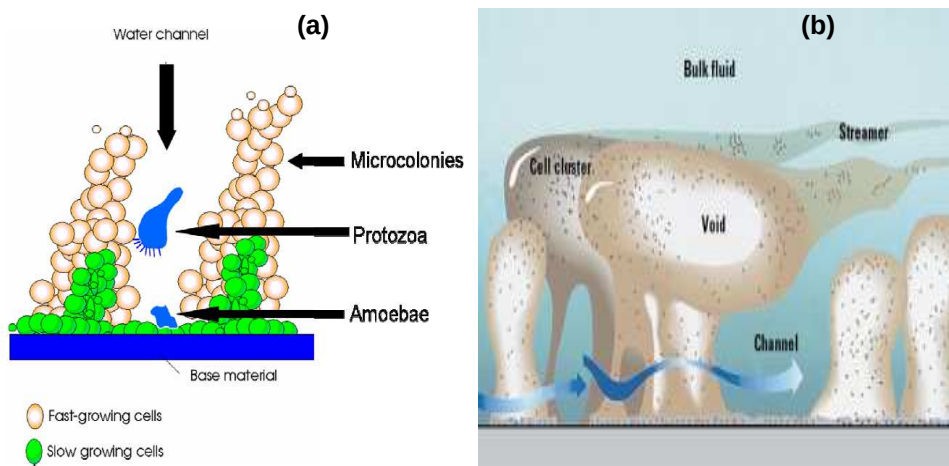


Figure 1.7 - Open architecture structure of biofilm with fronds, water channels as proposed by (a) - C. W. Keevil (1995) and (b) - W. Costerton (Source: Peg Dirckx, Center for Biofilm Engineering).

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 an 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 1.7b).

All these conceptual structure models may be possible to occur depending on the great variety of environment parameters such as the hydrodynamics, the nutrients composition, the temperature and the pH that, consequently, affect the density, the porosity and the thickness (Wimpenny and Colasanti, 1997; Sutherland, 2001b; Horn *et al.*, 2002). In some research works (Zhang and Bishop, 1994b, 1994a; Bishop *et al.*, 1995), heterogeneous biofilms with depths ranging from 52 to 1710 μm , presented densities 5-10 times higher in the top than in the bottom layers while the porosity in the top layers was much higher; also, the fractions of redox active cells and the cells/biomass ratios were much higher for the top newly layers. Most environmental biofilms were heterogeneous microbial communities that have different behaviors depending on the conditions (*e.g.*, exopolymers production) and interactions with each other (*e.g.*, chemotaxis, metabolic interactions), hence forming unique biofilms where all resources and energies optimized.

The distribution of a drinking water biofilm on pipe surfaces does not follow a standard rule: they may cover all the inner surfaces (Donlan, 2002; Chu *et al.*, 2003; Yu *et al.*, 2007) or be formed by dispersed aggregates (Keevil and Walker, 1992).

Paris *et al.* (2007) observed that drinking water biofilm was occupying only 7 % of the total glass surface and this coverage was independent from the applied wall shear rates. The surface coverage degree depends of many factors, such as, the type of microorganisms, biofilm age, hydrodynamic conditions, presence of inorganic particles, nutrients and temperature. As an example, Murga *et al.* (1995) grew biofilms of motile *Pseudomonas aeruginosa* and of non-motile *Klebsiella pneumoniae* and observed that the latter formed a patchy biofilm while the former built an uniform biofilm. SEM examination of biofilms grown by Harrison *et al.* (2006) revealed that different bacterial strains adopted various structural conformations. For instance, *Pseudomonas chlororaphis* PcO6 formed thick cell layers with high cell density whereas *Staphylococcus aureus* ATCC 29213 adhered in clumps. In another example, *Escherichia coli* JM109 formed uneven layers of single and multiseptate cells that were clustered into mounds. The multiseptate cells form chains of multiple cells connected one to another in biofilm as also happened during cell replication and division in suspension.

1.6.3 Biofouling

Biofouling occurs when biofilm accumulation on submerged surfaces is undesired as already observed in many industrial processes (*e.g.* food processing, paper industry, chemical process industry), medical and dental devices and drinking water systems (Mattilasandholm and Wirtanen, 1992; Barbeau *et al.*, 1998; Walker and Marsh, 2004). Biofilms can create problems for human health, hygiene and cleaning, as well as being responsible for energy losses, blockages in systems and microbially induced corrosion (Videla, 2001).

While some biofilms are detrimental, others may be beneficial, such as those used for the wastewaters treatment, the metals removal or to produce certain chemicals such as citric acid, ethanol, L-lysine, xanthan and alginate. Biofilms present in the intestinal tract are also beneficial as well as those used in microbial fuel cells (Logan *et al.*, 2005).

In drinking water systems, bacterial growth may affect turbidity, taste, odor and color of the distributed water (Lanciotti *et al.*, 2003; Rogers *et al.*, 2004; Vreeburg and Boxall, 2007). Another problem is the biofilm detachment to water decreasing the microbiological quality, especially if pathogens were present.

1.7 Factors affecting Biofilm Growth

Several factors promote bacteria growth in drinking water distributions systems, called regrowth, which occurs in the water phase and in biofilms on the pipe surfaces and reservoirs. The sections below provide an overview on the factors affecting regrowth in distribution systems.

1.7.1 Temperature and pH

Temperature and pH are considered two of the most important factors affecting life by modifying the electrostatic interactions between surfaces and microorganisms, enzymatic activity, kinetics and equilibrium of reactions, and many other properties (*e.g.* diffusivity, solubility). Although pH affects the metabolic activity, microorganisms withstand some variations in the pH as their own metabolic activities alter locally the pH by producing acids. Generally, bacteria have an optimal pH at which their growth is higher. A recent study (Chen *et al.*, 2005) concluded that attached *Pseudomonas fluorescens* accumulated at a higher extent and more cohesively in the biofilm at neutral pH than in other acid or basic media.

Chlorine residuals present in drinking water systems are drastically reduced when temperature increases and pH decreases. In North America (LeChevallier *et al.*, 1996), the coliform bacteria increased significantly in drinking water systems when the temperature increased to over 15 °C. After recognizing the importance of these two parameters, Portuguese water regulation authorities defined that temperature and pH should be always lower than 25 °C and 9.5, respectively.

1.7.2 Disinfectant Agents

The chemical disinfection and the maintenance of chlorine residual through the distribution system are worldwide accepted as efficient to prevent bacterial regrowth during the transportation (LeChevallier *et al.*, 1996; Momba *et al.*, 1998; Servais *et al.*, 2004). Even so, regrowth may occur considerably when chlorine residual decay further down in the distribution system (Chandy and Angles, 2001; Hallam *et al.*, 2002). Several disinfectant agents have been used, as described in section 1.4.

Bacteria embedded in the biofilm matrix have higher protection from the disinfectants (LeChevallier *et al.*, 1988a, 1988b; Bois *et al.*, 1997; Flemming, 2002). Disinfectant concentration was much lower within the biofilm than in the

water phase (de Beer *et al.*, 1994). Consequently, it actuated first in the outermost layer while bacteria were still metabolically active in the inner layers (Huang *et al.*, 1995).

Several mechanisms have been proposed to explain the resistance of microbes in biofilms against biocides. Extracellular polymers associated with biofilms prevent the penetration of biocides, protecting the microbes by limiting the diffusion or by reacting. Induction of stress responses and development of biofilm-specific biocide resistant phenotype may contribute to biocide resistance (Morton *et al.*, 1998). Moreover, the bacteria resistance was increased with the older biofilms and for the Gram-positive bacteria. Another surprising way of increasing the resistance against biocides was found out when observing pathogen bacteria (*e.g. Legionella pneumophila*, *Escherichia coli*, *Salmonella* and *Campylobacter*) hosted inside protozoa in drinking water distribution systems (Pedersen, 1990; Kalmbach *et al.*, 1997b; Sibille *et al.*, 1998; Keevil, 2003; Berry *et al.*, 2006; Snelling *et al.*, 2006).

During the disinfection of the drinking water systems, several harmful by-products are formed and taste and odor are affected (see section 1.4). Although biofilms promote the degradation of some haloacetic acids (Bayless and Andrews, 2008), several authors still defend that the disinfection by chemical oxidation should be reduced and applied coupled with other actions (van der Kooij *et al.*, 1999a; van der Kooij *et al.*, 1999b; Momba and Binda, 2002; Batté *et al.*, 2003c).

It should be pointed out that the disinfectant has different efficacies on killing the pathogens that were already contaminating the water supplies (Table 1.1). According to a report from the World Health Organization (WHO, 2006), the pathogenic bacteria, virus and protozoa have low, moderate and high resistance to chlorine, respectively.

1.7.3 Availability of nutrients

Generally drinking water distribution systems are considered oligotrophic environment with low contents of carbon, nitrogen and phosphorous. Several reports from the drinking water distribution systems in Sydney - Australia (Chandy and Angles, 2001), Paris - France (Servais *et al.*, 1995; Batté *et al.*, 2003b; Servais *et al.*, 2004), New Jersey - USA (LeChevallier *et al.*, 1987; LeChevallier *et al.*, 1991b), Singapore - Singapore (Hu *et al.*, 2005), Barcelona - Spain (Frias *et al.*, 2001), Nieuwegein - Netherlands (van der Kooij, 1992) and in Shanghai - China (Bai *et al.*, 2006) observed that the organic carbon content was the limiting nutrient

because an increase in this nutrient promoted bacteria regrowth and the addition of phosphorous did not affect its accumulation although phosphorous was being incorporated in biofilm (Batté *et al.*, 2003b). Other researchers observed that the limiting nutrient was the phosphorous in the drinking water from Tokyo - Japan (Sathasivan and Ohgaki, 1999) and Kuopio - Finland (Lehtola *et al.*, 2002). The detection of the limiting nutrient in drinking water systems is very important since the addition of phosphate based compounds has been proposed to prevent pipe corrosion and the bacterial regrowth (Szewzyk *et al.*, 2000; Appenzeller *et al.*, 2001; Appenzeller *et al.*, 2002).

Biofilm structure is affected by the nutrient concentration in solution, mass transfer and the rate of nutrients consumption (Tijhuis *et al.*, 1996; VanLoosdrecht *et al.*, 1997; Wimpenny and Colasanti, 1997). For example, the biofilm accumulation of *Pseudomonas fluorescens* increased with the glucose concentration, but the biofilm formed at the higher glucose concentration (45 mg/L) had a more fluffy, open and loose structure (Chen *et al.*, 2005). This resulted in a higher sloughing rate (due to the lower adhesive strength), as well as a larger sloughing biomass. The nutrients composition such as carbon/nitrogen ratio is important to the production of extracellular polymers and thus affected the adhesion of microorganisms to surfaces (Veiga *et al.*, 1997).

1.7.4 Hydrodynamic Conditions

The hydrodynamic conditions in drinking water systems range alternatively from laminar to turbulent flow, but stagnant (no-flow) waters also occur in places where the water consumption is low, as well as in reservoirs in buildings. The flow velocity may cause different effects (Melo, 2003) on biofilm accumulation and detachment. 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 rates promote bacterial growth within the biofilm. On the other side, the biofilm density and detachment increased with the velocity.

The hydrodynamic conditions and the nutrients are the two main parameters that influence biofilm growth in particular the structure, density and thickness (Tijhuis *et al.*, 1996; Wimpenny and Colasanti, 1997; Horn *et al.*, 2002; Wasche *et al.*, 2002). High shear stress and limitation of nutrients led to thin and dense biofilms that will have reduced internal nutrient diffusion (Zhang and Bishop, 1994b; Melo and Vieira, 1999) and increased resistant to removal and cohesion (Vieira *et al.*,

1993; VanLoosdrecht *et al.*, 1997; Picioreanu and van Loosdrecht, 2003; Chen *et al.*, 2005; Paris *et al.*, 2007). When low hydrodynamic shear stress and high nutrients content were applied, the biofilm grew extremely quickly and with a less dense structure with many pores, channels and protuberances (vanLoosdrecht *et al.*, 1995).

As a result of the different effects of the velocity, perhaps the conclusions of the experimental works are not as controversial as seemed at first glance. Several authors (Percival *et al.*, 1999; Lehtola *et al.*, 2006; Simões *et al.*, 2006; Paris *et al.*, 2007) observed that biofilm growth increased with the flow velocity while others (Soini *et al.*, 2002; Stoodley *et al.*, 2002; Chen *et al.*, 2005; Tsai, 2005) achieved the opposite effect.

A mechanistic explanation about the effects of the different hydrodynamic conditions was given by several metabolic studies (Tay *et al.*, 2000; Teo *et al.*, 2000; Liu and Tay, 2001a; Liu *et al.*, 2003). Higher flow velocity increases cells hydrophobicity that will favor cells aggregation and hence biofilm accumulation.

Alternation between flow/no-flow conditions is expected to affect not only biofilm accumulation as well as detachment from the surfaces deteriorating microbiological water quality. An increase of shear stress corresponded to higher detachment rates independently from the hydraulic conditions during biofilm growth (Choi and Morgenroth, 2003). Biofilms formed under alternating conditions behave like dynamic structures in which the density decreased with the shear stress. If the shear stress increased again then the detachment rate increased also. Similar conclusions were observed in another work (Ohl *et al.*, 2004).

Although **stagnancy** occurs in every distribution systems when the drinking water consumption is low, reports on drinking water biofilms under stagnant conditions in pipes and containers are not as numerous as under flow conditions. Le Chevalier *et al.* (1987) obtained high bacterial counts in drinking water after opening a tap that was turned off overnight. Momba and Kaleni (2002) studied the regrowth of microorganisms on household containers used for storage of drinking water and observed higher values of heterotrophic plate counts and DOC (dissolved organic carbon) after storing water for 48 hours. Ayoub and Malaeb (2006) observed also an enhancement of bacteria re-growth during the periods of no-flow in household tanks.

1.7.5 Surface Material

In the past, the majority of pipelines in drinking water networks were made of iron-based or cement-based materials. More recently, polymeric materials have been preferred, mainly PVC (polyvinyl chloride) and PE (polyethylene), because they are easier to handle and implement. In U.S.A. the majority of distribution system pipes are composed of iron material: cast iron (38 %), ductile iron (22 %), and steel (5 %) where corrosion is the most common problem (AWWA report, 1996). This corrosion will increase soluble iron in water, transport head loss and turbidity, and consequently consumer complaints of "red water" at the tap (McNeill and Edwards, 2001).

There is still some controversy about the effect of surface materials on biofilm development. Some researchers (Kerr *et al.*, 1999; Niquette *et al.*, 2000; Hem and Skjevrak, 2002; Momba and Kaleni, 2002; Chang *et al.*, 2003) demonstrated that drinking water biofilms grew less on polymeric materials (PE, PVC, PEX, Teflon) than on iron matrices (grey iron, cast iron, galvanized steel, stainless steel, cemented steel, asbestos-cement and cemented cast iron). This fact was attributed to iron corrosion products that favor biofilm protection from the effect of flow rate and of disinfectants. Different results were obtained by other researchers (Schwartz *et al.*, 1998; Cloete *et al.*, 2003; Bachmann and Edyvean, 2006) which reported higher biofilm formation on PVC and PE surfaces than on galvanized steel piping. In other works (Clark *et al.*, 1994; van der Kooij and Veenendaal, 2001) PE supported biofilm formation in a higher degree than PVC, while other works (Pedersen, 1990; Zacheus *et al.*, 2000; Wingender and Flemming, 2004) concluded that there was no significant difference in the colonization of the investigated materials (stainless steel, PVC and PE) after decades of operation. Lehtola *et al.* (2004) found that biofilms grew faster on PE than on copper pipes, but such differences could not be detected in older piping systems. The considerable inhibitory effect of copper was also observed (Mueller *et al.*, 1992; Schwartz *et al.*, 1998; Hem and Skjevrak, 2002) when compared to biofilm growth on other materials (HPDE, PVC, silicon, stainless steel and glass). Copper ions were found to cause inhibition of the respiratory chain of bacteria (Domek *et al.*, 1984), which may contribute to the low biofilm formation.

Roughness of the materials used in drinking water distribution system has been identified as an important factor affecting biofilm accumulation (Pedersen, 1990) shielding the microorganisms from shear stress on rougher surfaces and hence

producing a biofilm with higher cell densities and bacterial adhesion strength (Percival *et al.*, 1998). Although drinking water biofilm accumulated more on rough stainless steel than on smooth stainless steel, the differences disappear after 12 months of operation (Mueller *et al.*, 1992; Katsikogianni and Missirlis, 2004; Chen *et al.*, 2005).

Moreover, **corrosion** resistance may be another important factor when choosing the material for the water pipelines distribution. Corroded iron pipes may offer numerous bacterial attachment sites and bacteria protection from disinfectants as well as may release undesirable product to the water (LeChevallier *et al.*, 1993; Niquette *et al.*, 2000). Also, the corrosion products may retain nutrients (such as, humic matter) for subsequent utilization by biofilm bacteria (Butterfield *et al.*, 2002). The corrosion on metallic surfaces may be induced by the activity of physiologically diverse microbial species within the biofilms (Beech, 2004). These include the consumption of oxygen, production of acids, sulphides and enzymes that promote the establishment of localized chemical gradients at the metal surface. These chemical gradients may induce the development of electrochemical cells, thus affecting the anodic and/or cathodic reactions and probably leading to the loss of metal from the surface which will color the water (Schwartz *et al.*, 1998). Also, the interaction of the exopolymers with metal cations has long been proposed as one of the mechanisms of metal biodeterioration (Davey and O'Toole, 2000; Wotton, 2004).

Hydrophobicity and hydrophilicity. Although evident the effect of the surface/microorganisms thermodynamics on microbial adhesion, experimentally the results were controversial. There is still some controversy about the effect of the hydrophobic character of the materials on biofilm growth. Some authors (Pedersen, 1990; Tsuneda *et al.*, 2003) obtained no correlation between hydrophobicity and biofilm accumulation. In opposition, other works (Absolom *et al.*, 1983; Mueller *et al.*, 1992; Teixeira and Oliveira, 1999; Pereira *et al.*, 2000; Cerca *et al.*, 2005; Liu and Zhao, 2005; Allion *et al.*, 2006; Pereni *et al.*, 2006; Hadjiev *et al.*, 2007) demonstrated that biofilm adhesion and growth were explained by the surface free energies of several materials. These differences may be explained by the effect of other factors, besides the hydrophobicity, such as, the interactions between the complex physic and chemistry of both intervenient structures, which can be attractive or repulsive. Furthermore, the effect of the hydrodynamic conditions of biofilm growth “was emphasized” as very important when looking for correlation between the adhesion and the cohesion of biofilm and the biofilm thickness on a

certain surface (Brizzolara and Holm, 2006). According to this author this effect may help to explain the discrepancies in the existing literature regarding the effect of solid surface tension on the propensity of a biofilm accumulation. In the case of very low or null shear stress, the measured quantity of biofilm has a weak or no dependence on biofilm adhesion and cohesion as observed by several authors (Wiencek and Fletcher, 1995; Tegoulia and Cooper, 2002). In the case of high shear stress the measured quantity of biofilm has a strong dependence on the adhesion and cohesion of the biofilm (Brizzolara and Holm, 2006). Simões *et al.* (2007) concluded that the physico-chemistry of the surfaces can not explain alone the adhesion of bacteria isolated from drinking water to several surfaces.

Also, temperature, pH, water chemical composition and the physiological state and the type of microorganism will affect the attachment process (O'Toole *et al.*, 2000). Several theoretical approaches have been used to explain the adhesion equilibrium, although not always with success. These may indicate that either theories are incomplete (hence excluding important effects) or some assumptions are not fulfilled. For instance, these theories consider the microorganisms as colloidal particles with homogeneous surfaces. In fact, the bacterial surface is a highly heterogeneous and complex structure that might contain outer membrane proteins, capsule material / EPS, flagella, curli, fimbriae, that will interact in the bacterial attachment to the surface.

Nowadays, one of the most applied theories is based on the thermodynamic approach in which attachment is thermodynamically favored if the total free energy of a system containing a cell and an adjacent material is reduced by contact. This free energy of adhesion is dependent on the interfacial tension between the interfaces cell/material, material/liquid and cell/liquid. The thermodynamic approach is based on the theory of the contact angle of pure liquids on a solid surface expressed in the Young equation. According to van Oss methodology and the acid-base approach (van Oss and Giese, 1995), the surface free energy is composed by a Lifshitz-van der Waals apolar component and a Lewis acid-base polar component. The latter is separated in an electron donor and an electron acceptor component. The total free energy of hydrophobicity is then calculated using the different surface free energy, which is negative for hydrophobic materials and positive for hydrophilic materials.

Migrating components from surfaces. Another factor is the effect of pipe composition on densities of bacteria fixed on pipe walls by releasing volatile components. van der Kooij *et al.* (1995; 2002) also observed that the polymeric

materials in contact with drinking water could release biodegradable compounds, thus enhancing biofilm formation and affecting water quality. Several volatile components migrating from plastic pipes (HPDE, PEX and PVC) into drinking water were identified (Skjevrak *et al.*, 2003; Skjevrak *et al.*, 2005). A major migrating component from HDPE pipes was 2,4-di-tert-butyl-phenol (2,4-DTBP), which is a known degradation product from antioxidants. In addition, a range of esters, aldehydes, ketones, aromatic hydrocarbons and terpenoids were also identified. Corresponding migration tests carried out for PEX pipes showed that volatile organic compounds migrated in significant amounts into the test water, being the methyl-tert-butyl ether (MTBE) the predominant component. Several unidentified compounds, presumably various oxygenates were encountered in the test water samples. Migration tests of PVC pipes revealed few volatile migrants, such as hexanal, octanal, nonanal and decanal, in the samples with no significant odor.

The effect of **valves and joints materials** is also important for the biofilm accumulation. Biofilms have been observed to form at a higher degree on rubber, used in valves and joints, than on plastic and iron materials, and thus, contaminating drinking water (Wingender and Flemming, 2004). Coliform occurrences in several drinking waters have also been associated with dense biofilms containing coliforms located on rubber-coated valves, while biofilms on the pipe surfaces were negligible (Kilb *et al.*, 2003).

Pipe materials affect the disinfectant efficiency of biofilms. 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. A recent study demonstrated also that pipe service age was an 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 (Al-Jasser, 2007).

1.7.6 Protozoa Grazing

Protozoa are considered the major organisms responsible for bacterial grazing in aquatic environments, which has been shown to limit biofilm accumulation in

drinking water systems (Kalmbach *et al.*, 1997b; Sibille *et al.*, 1998; Brown and Barker, 1999; Percival *et al.*, 1999; Berry *et al.*, 2006; Snelling *et al.*, 2006). In contrast to predation, association of several pathogenic bacteria to protozoa is well documented as the latter have high resistance to chlorine; hence promoting resistance against disinfectants and increasing the health risk events (see section 1.7.2).

1.8 Kinetics of Biofilm Accumulation

There is a lack of information on the kinetics of bacterial growth in drinking water biofilms as compared to suspended bacteria. Authors studying wastewaters and industrial waters are divided between those who defend that the activity of sessile and planktonic bacteria is similar (Bakke *et al.*, 1984; Alldredge *et al.*, 1986; Characklis and Marshall, 1990; Kiorboe *et al.*, 2003) and those who reached opposite conclusions (Plas *et al.*, 1984; Le Magrex *et al.*, 1994; Ploug and Grossart, 2000).

Quantitative data on bacterial growth kinetics in drinking water systems is scarce (van der Wende *et al.*, 1988; Pedersen, 1990; Block *et al.*, 1993), although such data would seem important for the proper design of distribution networks and reservoirs. The values of biofilm specific growth rates reported by these authors range between 0.03 day^{-1} and 0.06 day^{-1} .

Furthermore, in different systems, the average specific growth rates of cells in the biofilm were much lower than those in suspension according to the published literature in the last decade (Wiesmann, 1994; Lappin-Scott and Costerton, 1995; Vieira and Melo, 1999). Davies and McFeters (1988) reported that cells of *Klebsiella oxytoca* attached to granular activated carbon particles had a growth rate 10 times higher than cells in suspension. Alldredge *et al.* (1986) achieved a different result: attached bacteria were growing slower or equally to the free-living bacteria.

1.9 Microorganisms in Drinking Water Systems

Microbial Composition. In drinking water and biofilms, *Acinetobacter*, *Alcaligenes*, *Arthrobacter/Corynebacterium*, *Bacillus*, *Flavobacterium*, *Methylobacterium*, *Moraxella*, *Pseudomonas*, *Staphylococcus*, *Mycobacteria* and *Sphingomonas* have been the predominant genera detected (LeChevallier *et al.*,

1987; Rogers *et al.*, 1994; Percival, 1999; Percival and Walker, 1999; Koskinen *et al.*, 2000; Penna *et al.*, 2002; Torvinen *et al.*, 2004; Bachmann and Edyvean, 2005). All studies reported that Gram negative were predominant over the Gram positive bacteria.

Nitrification processes can be very important to drinking water-related companies' management strategies, because they affect the chloramines residual. In a chloraminated system, several authors (Wolfe *et al.*, 1990; Regan *et al.*, 2002; Martiny *et al.*, 2003; Lipponen *et al.*, 2004; Hoefel *et al.*, 2005a; Martiny *et al.*, 2005) detected ammonia- and nitrite-oxidizing bacteria (AOB and NOB, respectively) such as, *Nitrosomonas* and *Nitrobacter* belonging to the *Betaproteobacteria* and *Alphaproteobacteria*, respectively, and the genus *Nitrospira*.

Filamentous fungi and microfungi were also observed within the drinking water systems (Doggett, 2000; Codony *et al.*, 2003; Goncalves *et al.*, 2006; Paterson *et al.*, 2006; Kanzler *et al.*, 2007).

There are some published works where no pathogens were detected in drinking water systems. A study of biofilms accumulated on real pipe materials with up to 99 years old did not detect neither pathogenic bacteria (*Yersinia* spp., *Campylobacter* spp., *Helicobacter pylori* and colon cell-adhesive aeromonads) nor protozoan parasites such as *Giardia* and *Cryptosporidium* (Wingender and Flemming, 2004). Atypical mycobacteria, aeromonads, and free living amoebae, all with low infectious potential, were found occasionally. Similar conclusions were achieved by Schmeisser *et al.* (2003) while studying the microbial diversity of drinking water biofilm: a high diversity of bacteria was observed, but no pathogens were detected. No *Pseudomonas aeruginosa* was detected in biofilm samples collected from the drinking water system in Harbin-China (Yu *et al.*, 2007).

In other published research works, pathogens were detected in drinking water systems. *Giardia* and *Cryptosporidium* cysts/oocysts were detected in 17 % and 39 % of the drinking water samples in USA and Canada (LeChevallier *et al.*, 1991a). *Legionella pneumophila* was isolated from 30 % of water samples taken from the distribution systems of buildings in Finland (Zacheus and Martikainen, 1994). When samples of hospital water systems were taken in USA, Canada and Great Britain, 12 to 70 % were colonized with *Legionella* (Lin *et al.*, 1998). Potentially pathogenic mycobacteria were detected in 16.3 % of water samples collected in France (LeDantec *et al.*, 2002). *Mycobacterium* spp. was isolated from 5 to 32 %

samples of drinking water and from 17 to 100 % of biofilms (Falkinham *et al.*, 2001). Infectious enteroviruses and adenoviruses were detected in 47.8 % and 39.1 % of the tap water samples, respectively, collected in urban sites in Korea (Lee and Kim, 2002). Opportunistic pathogens, mycobacteria, *Legionella* spp. and *Pseudomonas aeruginosa*, were detected in biofilms and drinking water in Germany (Emtiazi *et al.*, 2004). *Cryptosporidium* spp. oocysts were detected in drinking water samples in United Kingdom (Nichols *et al.*, 2003). In Russia and Bulgaria, 10 % and 30 % of water samples were positive for *Giardia* and *Cryptosporidium* (Karanis *et al.*, 2006). Enteroviruses were detected in 11 and 16 % of drinking water samples collected from two treatment plants in South Africa (Vivier *et al.*, 2004).

Using direct sequence analysis of 16S rDNA clone libraries, the greatest diversity in drinking water from a distribution system simulator fed with drinking water in USA belonged to *Alpha*-, *Beta*- and *Gammaproteobacteria* being *Alpha*- and *Beta*- the dominant sub-classes in both chloraminated and chlorinated water (Domingo *et al.*, 2003; Williams *et al.*, 2004; Williams *et al.*, 2005). Polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) revealed that *Betaproteobacteria* were also abundant in biofilms of non-chlorinated drinking water (Emtiazi *et al.*, 2004). These studies indicate that microbial community diversity is affected by the disinfection strategy. For example AOB densities increased when chloramine was added and decreased after the disinfection treatment was switched back to chlorination. Tokajian *et al.* (2005) observed that *Alpha*-, *Beta*-, and *Gammaproteobacteria* were found to be the major groups representing the heterotrophic bacteria in drinking water.

Using Fluorescent *in situ* Hybridization (FISH), the most frequent bacteria found within biofilm population from drinking water distribution system in Germany belonged to the *Beta*- and *Gamma*-subclass of *Proteobacteria* (Schwartz *et al.*, 1998) while in other distribution system only bacteria belonging to the *Betaproteobacteria* were dominating in the community (Kalmbach *et al.*, 1997b, 1997a). In biofilms collected from a German drinking water reservoir, the most abundant groups were the *Alpha*- and the *Betaproteobacteria* (Uhlmann *et al.*, 1998). The other phylogenetic groups identified were *Cytophaga-Flavobacterium* group, sulfate reducers and Gram-positive bacteria with a high G+C content, *Gammaproteobacteria* and *Nitrosomonas*.

Physiological State of Microorganisms. Some bacteria from oligotrophic water systems were unable to form colonies on commonly used media but were still

viable. Different physiological states were postulated (Kalmbach *et al.*, 1997a): (i) bacteria were cultivable but incapable of undergoing the sustained cellular division required for growth on artificial medium; (ii) bacteria were not cultivable on that medium. According to Garland *et al.* (2001), the cultivability capacity of bacteria may decrease due to a shift in resources allocation from growth to maintenance. Generally only a small fraction of all bacteria in drinking water systems are able to form colony units. Wingender and Flemming (2004) observed that the cultivable cells were at most 3.5 % of the total cells in biofilms aging from 2 to 99 years. Boe-Hansen *et al.* (2002b) reported that the highest percentages (up to 24 %) were observed in the youngest biofilms in an assay of 522 days.

The metabolic state of the cells in suspension and in biofilms is also an important variable since it can be a measure of their growing potential and, therefore, of the risk of water contamination. Schwartz *et al.* (1998) studied the development of biofilms on HDPE (high density polyethylene), PVC, steel and copper in contact with drinking water collected at the water works and in house branch connections: the biofilm densities of cultivable bacteria on the different materials were about one order of magnitude less than the total cell number; the metabolic active bacteria were from 35 to 38 % except for copper, where they were less than 10 %. Schaule *et al.* (1993) also found that the percentage of metabolic active bacteria in biofilms sampled within the distribution system was about 5 - 35 %. In drinking water the fractions were lower than those found in the biofilm: around 1 - 10 % of total cells possessed metabolic activity.

Effect of Disinfection on Microorganisms. The effect of disinfection on the microorganisms is much more efficient against Gram-negative and thus, microbial communities were shifted to predominantly Gram-positive bacteria after rechlorination (Norton and LeChevallier, 2000). The type of disinfectant (free chlorine, chlorine dioxide and monochloramine) was observed to affect bacterial populations in drinking water biofilms that changed in response to disinfection practices (Schwartz *et al.*, 2003a; Williams *et al.*, 2005). In the absence of exposure to chlorinated water, most biofilm bacteria are Gram negative and have an outer layer that contains endotoxin which, by hemodialysis and inhalation, has been attributed as the cause of several drinking-water-associated incidents (Anderson *et al.*, 2002). Eicher *et al.* (2006) observed also that chlorination in drinking water systems enhanced nitrifying bacteria. In a comparison of chlorinated and chloraminated distribution systems, losses in chloramine level owing to nitrification (measured by the increase in nitrate) led to increased overall microbial growth

(Zhang and DiGiano, 2002). It appears that AOB are present in chloraminated systems irrespective of temperature fluctuations and can be controlled only through very high or very low chloramine levels (Pintar and Slawson, 2003).

Effect of Pipe Materials on Microorganisms. Microbial biofilm composition is dependent on the type of pipe material as was observed elsewhere (Kerr *et al.*, 1999; Norton and LeChevallier, 2000). Although the predominant phylogenetic group (*Betaproteobacteria*) was similar, the percentages were different for the several tested materials (HDPE, PVC, iron, glass and copper) (Kalmbach *et al.*, 1997b, 1997a; Schwartz *et al.*, 1998). Kalmbach *et al.* (1997b) observed that these differences were reduced with the biofilm lifelength.

Effect of velocity on microorganisms. Several works (Percival *et al.*, 1999; Liu *et al.*, 2006) showed that fluid velocity affected the microbial composition within biofilms. At low flow velocities, *Acinetobacter* spp. was observed as being the principle pioneering bacterium, together with *Corynebacterium/Arthrobacter* spp. At higher velocities, the pioneering bacterium *Acinetobacter* spp. was also present with *Pseudomonas* spp. Also the pathogen *Legionella pneumophila* accumulated much more in a biofilm of a simulated water system in turbulent flow than in laminar flow.

1.10 Biofilm Monitoring Reactors

Laboratory research has been performed in a wide variety of systems or “biofilm monitoring reactor” that mimics the *in situ* situations with reproducible results; thus important information is assessed about microorganisms’ behavior within the real drinking water systems.

The Robbins device was one of the first biofilm reactors patented. It consists of a cylinder which may be inserted as a bypass into a pipe of a water distribution system. The surface of the removable metal studs forms an integral part of the metal surface available for bacteria colonization.

Flow cells, basically modified Robbins device, are arguably one the best recognized in the area of drinking water biofilms (Pereira *et al.*, 2002a). This flow cell 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 unit several coupons, with the adhesion materials, are attached to the inner surface and may be replaced gradually without affecting the remaining system.

Propella reactor has more recently arisen (Batté *et al.*, 2003b) and 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.

Other reactors have also been extensively used such as: the rotating annular reactors, known as Rotatorques, in which the inner drum is rotated to provide shear stress; the flat plate flow cells used for *in situ* microscopic observations or analysis (such as, by FTIR); the chemostats, also known as the Continuously Stirred Tank Reactor (CSTR) in which nutrients are constantly fed in. There are other reactors where water is not allowed to recirculate and the hydraulic conditions approach the plug flow such as the ones used by Boe-Hansen *et al.* (2002a) and Lethola *et al.* (2004).

Also, biofilm accumulation and adhesion had been studied under no flow conditions in batch reactor meaning that the microbial suspension remains stagnant and no mass exchanges occur during the experiments.

The fact of using different reactors with different geometries and features may explain partially the conflicting data reported about biofilm growth, structures and behaviors as well as increases the entropy when comparing results between research groups (Bachmann and Edyvean, 2005).

2. Materials and Methods

2.1 Introduction

This chapter intends to overview all the experimental methodology used in this study. The biofilm monitoring reactors and the experimental set-up are presented. Propella and Flow Cell reactors were used to simulate the dynamic operating conditions similar to the ones observed in drinking water distribution systems. A batch reactor was also used to follow bacteria accumulation in stagnant water as it occurs when drinking water demand is too low, in distribution reservoirs or household containers. The methods applied in the analysis of biofilm and water samples and in the characterization of the adhesion surfaces are divided in three major sections: physicochemical, microscopic and microbiological methods. Growth kinetics parameters of bacteria attached on surfaces or suspended in reactor water are presented for steady state conditions in flow reactors. Calculation procedures and statistical analysis applied in the result treatments are also described.

2.2 Biofilm Monitoring Reactors

In the present work, the tested monitoring systems were a Propella reactor, a Flow Cell reactor and a Batch reactor.

2.2.1 Propella Reactor

Propella reactor (Figure 2.1, see description in section 1.10) was made essentially of PVC and allowed to position 20 screwed biofilm sampling points in the inner reactor surface. On each sampling point, a circular coupon of a surface material was glued (Figure 2.2), where biofilm accumulation was followed.

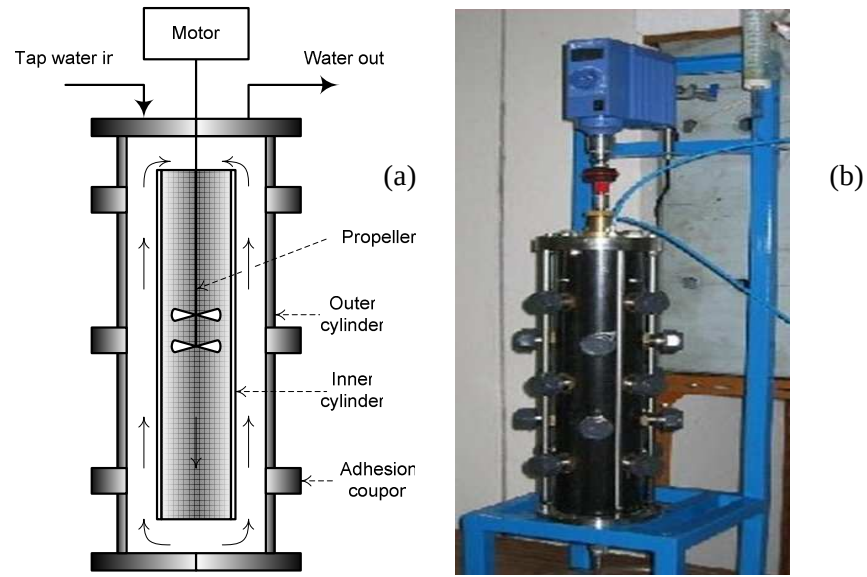


Figure 2.1 - Scheme (a) and image (b) of Propella reactor.

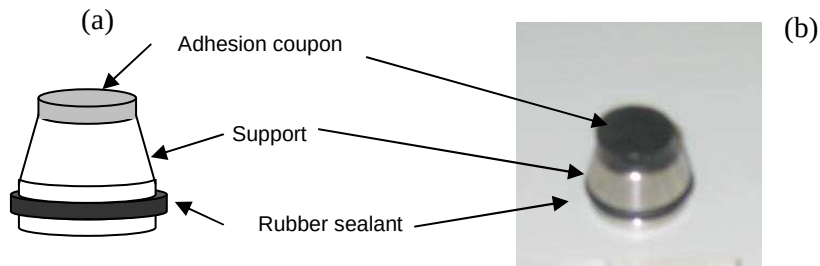


Figure 2.2 - Scheme (a) and image (b) of an adhesion coupon of Propella reactor used to collect biofilm samples.

The reactor dimensions are indicated in Table 2.1. The flow rate inside the pipe was controlled by the rotation speed of the propeller defined by the following equation:

$$u(\text{m/s}) = 1.48 \times 10^{-4} \omega(\text{rpm}) - 5 \times 10^{-3} \quad (2.1)$$

where u is the average velocity and ω is the angular velocity. The hydraulic residence time (HRT) is controlled by a peristaltic pump connected to the inlet pipe

and is proportional to the fresh inlet flow rate ($HRT = V / Q$). More detailed features of this reactor can be found in (Batté *et al.*, 2003c).

Table 2.1 - Characteristics of the Propella reactor.

Parameters	Propella reactor
Material	PVC and stainless steel 316 L
Internal cylinder dimensions (mm)	$D_{in} = 44$; $D_{ext} = 72.5$; $h = 460$
External cylinder dimensions (mm)	$D_{in} = 93.4$; $h = 500$
Internal Volume (L)	2.23
Volume/Surface Area (cm)	0.67
Biofilm sampling points	20
Biofilm sampling area (cm ²)	1.8

D_{in} - internal diameter; D_{ext} - external diameter; h - height.

2.2.2 Flow Cell Reactor

The Flow Cell reactor (Figure 2.3, see description in section 1.10) contained 10 sampling points on its flat surface in which a selected surface material was stacked to the support that was screwed to the cell wall (Figure 2.4). Its main characteristics are indicated in Table 2.2. The complete experimental set-up was composed by the Flow Cell, vessel F and recirculating tubes. The peristaltic and the centrifuge pumps were controlling the internal velocity and the residence time, respectively.

When biofilm accumulation was studied under similar conditions as those observed inside drinking water networks, the Flow Cell was connected directly to the tap. Two or three Flow Cells were also mounted in series or in parallel when more biofilm sampling points were needed.

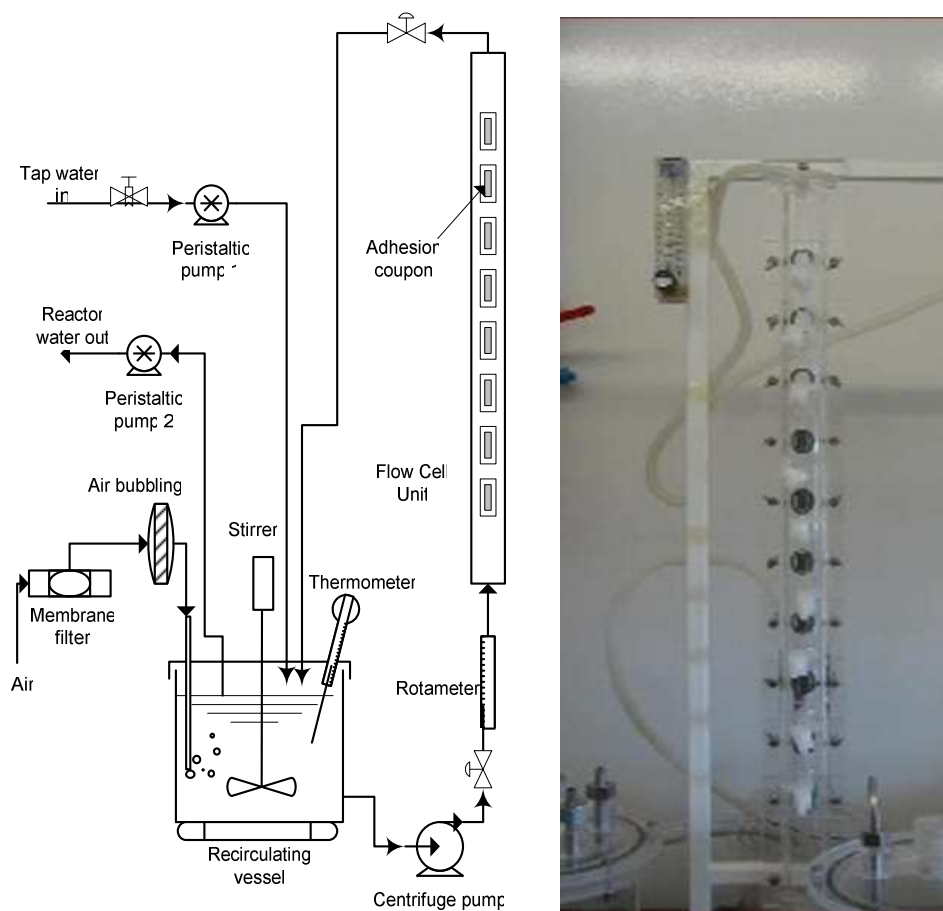


Figure 2.3 - Scheme (a) and image (b) of Flow Cell reactor.

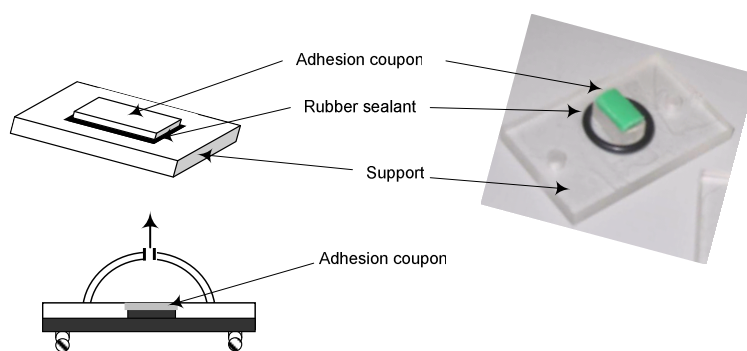


Figure 2.4 - Scheme and image: (a) - an adhesior coupon used to collect biofilm samples and (b) - the cross-sectional area of Flow Cell reactor.

Table 2.2 - Characteristics of the Flow Cell reactor.

Parameters	Flow Cell reactor
Material	Perspex (PMMA)
Dimensions (cm)	$D_{\text{int}} = 3.2$; $h = 85$
Internal Volume (L)	0.34
Volume/Surface Area (cm)	1.25
Biofilm sampling points	10
Biofilm sampling area (cm ²)	2.0

D_{in} - internal diameter; D_{ext} - external diameter; h - height.

2.2.3 Batch Reactor

The Batch reactor was a non-stirred cylindrical glass vessel operating as a closed system (Table 2.3). Twenty coupons, with dimensions of $1 \times 2 \times 0.5$ cm, were immersed in the reactor water in a vertical position, at 2 cm to 7 cm from the bottom, suspended by plastic wires in the central part of the vessel. The contact area between the plastic wire and the coupon was around 0.03 cm^2 , which corresponds to 0.6 % of the total area.

Table 2.3 - Characteristics of the Batch reactor

Parameters	Batch reactor
Material	Glass
Dimensions (cm)	$D_{\text{int}} = 12$; $h = 13$
Internal Volume (L)	1.50
Volume/Surface Area (cm)	2.41
Biofilm sampling points	20
Biofilm sampling area (cm ²)	4.9

2.2.4 Adhesion Surface Materials

Biofilms were formed on adhesion coupons made of several materials placed within the reactors in contact with drinking water under different operating conditions, including stagnant or flow regimes. Some of the tested coupons were made of polymeric materials that are common in pipelining construction: PVC (polyvinyl chloride), PEX (cross-linked polyethylene), HDPE (high density polyethylene) and PP (polypropylene).

Additionally, other materials were tested, namely several modified stainless steels whose surface treatments that were developed in the European project MODSTEEL while researching for surfaces less prone to milk fouling (Santos *et al.*, 2004; Rosmaninho *et al.*, 2007). The surface modifications tested for the stainless steel surfaces were prepared by Gerahard Rizzo (University of Stuttgart) and are the following:

- Diamond-like carbon (DLC) surface obtained by sputter coating of graphite targets;
- Silica surface (SiO_x) obtained by plasma enhanced chemical vapor deposition;
- Ni-P-PTFE coating obtained by autocatalytic electroless plating;
- Excalibur coating is composed by fluoropolymers and reinforced with stainless steel particles.

2.2.5 Cleaning of Reactors

Before each experiment, the reactors were cleaned with a chlorine solution and then with a mild detergent solution (non-ionic, neutral pH, phosphate-free - 25% Aquet, SCIENCEWARE). Finally, the reactors were rinsed abundantly with tap water and then with sterile deionized water.

2.2.6 Biofilm and bulk water Sampling

Biofilm samples were collected by removing test coupons from the reactors. The coupons were then immersed in a flask containing 25 mL of sterile bacteria-free saline solution 0.85 % wt. NaCl. The flask with the coupon was gently sonicated for 10 min in a sonication bath Transsonic 420 ELMA, 70 W, 35 kHz to release the attached bacteria into the suspension. The percentage of cells not detached from the surface by sonication was less than 0.3 %. At the same time, 50 mL of the water inside the reactors and of the inlet drinking water flow were collected to a sterile flask.

2.3 Physicochemical Methods

2.3.1 Temperature, pH and Total Dissolved Solids

Temperature measurements were performed with a mercury thermometer with an accuracy of 0.1 °C.

The pH values were measured with a pH meter (HANNA HI991003) at 25° C. Calibrations were made immediately before the measurements with standard solutions.

Total dissolved solids (TDS) were obtaining after multiplying the conductivity by a factor of 0.67 and the electrical conductivity was measured with a WTW LF 330/SET with 1 % of accuracy.

2.3.2 Total and Dissolved Organic Carbon

The total organic carbon (TOC) and the dissolved organic carbon (DOC) were determined for the water samples following the procedures described in the European Standard CEN 1484 and in Standard Method 5310 B. TOC values were obtained in triplicate by subtracting the values of the total carbon (TC) and the inorganic carbon (IC) measured in a non-dispersive infrared gas SHIMADZU Analyzer (TOC-5000A). Calibration curve for TC was performed in the range 0.5 - 10 mg C/L with standard solutions of potassium hydrogen phthalate in carbon free water. For IC, the calibration curve was obtained from standard solutions of sodium hydrogen carbonate + sodium carbonate in carbon free water in the same range. The detection limits for IC and TC were 0.34 and 0.37 mg C/L, respectively.

The dissolved organic carbon (DOC) was obtained following the described procedure with the samples pre-filtered through a 0.45 µm glass fiber membrane (ALBET).

2.3.3 Free and Total Chlorine

Free and total chlorine content was determined by the DPD – colorimetric method (Standard Method 4500-Cl G). Chlorine liberates iodine from potassium iodide at pH 4 or less. The iodine reacts with N,N-diethyl-p-phenylene diamine (DPD) to produce a red colored solution which is spectrophotometrically detected at 515 nm in a UV/VIS spectrophotometer (model Pye UNICAM 8600, PHILIPS). The

measured absorbance allows the calculation of the chlorine concentration through the calibration curve. Standard curve performed with a series of standards of potassium permanganate in the range from 0.2 to 4 mg Cl/L. The detection limit was 0.07 mg Cl₂/L.

2.3.4 Total Phosphorous

Total phosphorous was determined by the ascorbic acid method (SM 4500-P E) in a UV/VIS spectrophotometer (model Pye UNICAM 8600, PHILIPS). Ammonium molybdate and potassium antimonyl tartate react in acid medium with orthophosphate to form a heteropoly acid – phosphomolibdic acid – that is reduced to intensely colored molybdenum blue by ascorbic acid measured at 880 nm. Standard curve performed with a series of standards of KH₂PO₄ in the range 0.05-0.5 mg P/L. The detection limit was 0.01 mg P/L.

2.3.5 Nitrogen

Nitrogen may be present in different forms (NO₃⁻, NO₂⁻, NH₃ and organically bonded) and was evaluated separately and as total value by oxidation of all nitrogenous compounds to nitrate. The total nitrogen was performed according to the persulfate colorimetric method (SM 4500-N C) in which all nitrogenous compounds are oxidized to nitrate in alkaline medium at 110° C. The calibration curve was obtained from a series of standard solutions of KNO₃ of in the range of 5 to 50 mg NO₃⁻ / L and the detection limit was 0.7 mg N/L.

Nitrate content was obtained by the colorimetric method that uses brucine sulfate (SM D992-71) and the developed color intensity was measured at 410 nm in a UV/Vis spectrophotometer (UNICAM PU 8600). The calibration curve was obtained from a series of standard solutions of KNO₃ of in the range of 5 to 50 mg NO₃⁻ / L and the detection limit was 1.77 mg NO₃⁻ / L.

Nitrite was determined through the formation of a reddish azo dye produced in acidic medium with sulphanilamide (SM 4500 NO₂⁻ B). The optical density was measured at 537 nm in a UV/Vis spectrophotometer (UNICAM PU 8600). A calibration curve was obtained from a series of standards of anhydrous NH₄Cl in the range of 0.033 to 0.8 mg NO₂⁻ / L and the detection limit was 0.01 mg NO₂⁻ /L.

Ammonia was determined with a selective electrode (ORION 9512) (SM 4500 - NH₃ D). The calibration curve was performed with the proper ORION standards and the detection limit was 0.1 mg NH₃/L.

2.3.6 Contact Angles

Contact angles were quantified by the sessile drop technique using the equipment System OCA (DataPhysics Instruments). Three liquids with different polarities were used: water (Merck 1.01262), formamide (Merck 1.09684) and α -bromonaphthalene (Merck 8.06210). Materials surface were previously cleaned in a 2% wt. mild detergent solution (RBS35, RBS Chemical Products) at 65°C for 10 min followed by immersion in deionized water at 65 °C for 10 min and then water rinsed and air dried. The contact angles were performed after placing the liquid drop on the surface and then capturing automatically the drop image with a video camera to the software SCA20 v2.3.9 (DataPhysics Instruments). The values of the contact angles with water (θ_w), formamide (θ_F) and α -bromonaphthalene (θ_B), were obtained from 10 measurements for each probe liquid made at 25 °C. The liquid surface free energy components, γ_L^{LW} , γ_L^+ , γ_L^- , and the total surface free energy γ_L^{TOT} are presented in Table 2.4.

Table 2.4 - Lifshitz–van der Waals component (γ_L^{LW}) and electron-acceptor (γ_L^+) and electron-donor (γ_L^-) components and the total surface free energy (γ_L^{TOT}) of probe liquids at 20° C.

Liquid	Surface free energy (mJ.m ⁻²)			
	γ_L^{LW}	γ_L^+	γ_L^-	γ_L^{TOT}
Water	21.80	25.50	25.50	72.80
Formamide	39.00	2.28	39.60	58.00
α -bromonaphthalene	44.40	0.00	0.00	44.40

The surface free energy components of the surfaces, γ_S^{LW} , γ_S^+ and γ_S^- , were calculated according to the Acid-Base method and van Oss approach (van Oss, 1997), using the three equation system stemming from the application of Young-Dupré equation for each probe liquid:

$$\gamma_L^{\text{TOT}}(1 + \cos \theta_L) = 2 \left(\sqrt{\gamma_S^{\text{LW}} \cdot \gamma_L^{\text{LW}}} + \sqrt{\gamma_S^+ \cdot \gamma_L^+} + \sqrt{\gamma_S^- \cdot \gamma_L^-} \right) \quad (2.2)$$

Then the total surface energy γ_S^{TOT} and the polar component γ_S^{AB} were calculated by

$$\gamma_S^{\text{TOT}} = \gamma_S^{\text{LW}} + \gamma_S^{\text{AB}} \quad (2.3)$$

$$\gamma_S^{\text{AB}} = 2 \sqrt{\gamma_S^+ \cdot \gamma_s^-} \quad (2.4)$$

The software also computes the nonlinear correlation coefficient (RQ) which gives a measure how well the data is predicted by the applied model ($0 \leq \text{RQ} \leq 1$).

The hydrophobicity of a material (s) may be quantified (van Oss, 1997) in terms of the variation of the free energy of interaction between two moieties of that material immersed in water (w). The total free energy comprises an apolar (LW) and a polar component (AB), defined by:

$$\Delta G^{\text{TOT}} = \Delta G^{\text{LW}} + \Delta G^{\text{AB}} \quad (2.5)$$

$$\Delta G^{\text{LW}} = -2 \left(\sqrt{\gamma_s^{\text{LW}}} - \sqrt{\gamma_w^{\text{LW}}} \right)^2 \quad (2.6)$$

$$\Delta G^{\text{AB}} = -4 \left(\sqrt{\gamma_s^+ \gamma_s^-} + \sqrt{\gamma_w^+ \gamma_w^-} - \sqrt{\gamma_s^+ \gamma_w^-} - \sqrt{\gamma_w^+ \gamma_s^-} \right) \quad (2.7)$$

2.3.7 Surface Roughness

The surface roughness of the clean materials was measured using a surface profilometer PERTHEN SGP (CATIM). Five measurements were performed for each material over a specified sampling length and four statistical height-descriptive parameters were recorded (DIN EN ISO 4287-88). The roughness average (Ra) is the arithmetic average height in absolute values of roughness irregularities measured from a mean line within the sampling length.

$$\text{Ra} = \frac{1}{\ell} \int_0^\ell |Z(x)| dx \quad (2.8)$$

in which $Z(x)$ is the profile of ordinates of the measured roughness and ℓ is the sampling length. Rq is the geometric average roughness component irregularities

measured from the mean line within the sampling length and it is more sensitive to occasional and extreme peaks.

$$Rq = \sqrt{\frac{1}{\ell} \int_0^{\ell} Z^2(x) dx} \quad (2.9)$$

In each measured profile, the sampling length is divided in 5 evaluation lengths in which 5 roughness depths are determined (R_{zi} , $i = 1, 5$), that corresponds to the distance between the highest peak and deepest valley within each evaluation length. The mean roughness depth (R_z) and the maximum roughness depth (R_{max}) are then calculated according the following equations.

$$R_z = \frac{1}{5} \sum_{i=1}^5 (R_{zi}) \quad (2.10)$$

$$R_{max} = \max\{R_{zi}, i = 1, 5\} \quad (2.11)$$

The average and the standard deviation of the roughness parameters described above were calculated from 5 different measurements recorded in different areas of the surface.

2.4 Microscopic Tools

2.4.1 Epifluorescence (EF) Microscopy

An epifluorescence microscope is basically a conventional episcopic light microscope with a much higher intensity light that will excite fluorescence species present in the sample, and consequently emit light of a longer wavelength. This emitted light will be captured to produce a magnified image.

The EF microscope used is a LEICA DMLB2, with a mercury lamp HBO/100W/3, incorporating a CCD camera to acquire images using IM50 software (Leica). The characteristics of the filter cubes for the different fluorochromes are presented in Table 2.5.

An artifact (C-mount) was introduced in the microscope in order to acquire a higher amplified image by $\times 1.3$. The image acquisition system was calibrated with a micrometer ruler of 5 mm (+100/100) and checked every time the microscope set-up was modified. For 1000 magnification, 1 pixel corresponds to 6.46×10^{-6} cm. Without C-mount 1 pixel corresponded to 7.30×10^{-6} cm

Table 2.5 - Characteristics of the LEICA filter cubes used for each fluorochrome.

Fluorochrome	Filter cube	Excitation range	Excitation Filter (nm)	Dichromatic mirror (nm)	Suppression filter (nm)
DAPI	A	UV	BP340-380	400	LP425
FLUOS	I3	Blue	BP450-490	510	LP515
CTC	N2.1	Green	BP515-560	580	LP590
Cy3	Y3	Green	BP545/30	565	BP610/75
DAPI	B/F/G	UV	BP420/30	415	BP465/20
+FLUOS		Blue	BP495/15	510	BP530/30
+CY3		Green	BP570/20	590	BP640/40

2.4.2 Episcopic Differential Interference Contrast (EDIC) Microscopy

EDIC microscope was developed in the University of Southampton (Walker *et al.*, 1994; Keevil *et al.*, 1995; Keevil, 2003) and is composed of an adaptation of the Nomarski differential interference contrast (DIC) implanted in a normal light epifluorescence microscope. The EDIC/EF microscope has been shown to be particularly useful tool to observe structures and composition of biofilm grown on opaque surfaces (Keevil and Walker, 1992; Best and Keevil, 2005).

The modifications that were introduced in the EF microscopy were: a DIC filter block (containing the excitation filter, dichroic mirror and barrier filter), an adjustable prism analyzer and the non-immersion objectives. With these new features, opaque surface structures can be visualized well episcopically without placing coverslips or immersion oil that might distort their structure and interfere with the reflected episcopic light. Although the maximum magnification allowed is 1000, biofilm structures can be observed in the natural wet state as well as the biological components that can be fluorescently stained and visualized in the same viewing field.

2.4.3 Scanning Electron Microscopy (SEM/EDS)

The Scanning Electron Microscopy observations and the Energy Dispersive Spectroscopy (SEM/EDS) were performed in the Materials Center of University of Porto (CEMUP) with a JEOL JSM-6301F microscopy coupled with the micro-analysis system OXFORD INCA/ENERGY 350. This micro-analysis improves the surface structures observation and interpretation by allowing the quantification of elements the surface composition. The sample preparation of the surface materials and of the biofilms is described above.

Surface materials. All materials were left in an exsiccator for 2 to 5 days prior sampling preparation for SEM. After a step of CO₂ critical point drying, the surfaces that were not electron conductors (the polymerics and the surface modified materials) were sputter-coated with Au (100 Å of thickness). This thin Au-coating improves drastically the captured images by increasing the conductivity and the emission of secondary and backscattered electrons although some care should be taken when observing structures with dimensions similar to coating depth. The SEM observations were performed with different magnifications (5 000 - 15 000), at a working distance of 15 mm, with an energy beam of 10 - 15 keV. Similar energies were used for the elemental micro-analysis. The volume analyzed for the composition depends on the emitted electrons energy, elements composition and structures, but it may be considered that the analysis depth is surely higher than several micrometers.

Biofilm samples. Drinking water biofilms grown on the different surface materials were prepared by fixation with glutaraldehyde and dehydration with ethanol (Azeredo, 1998). Glutaraldehyde binds to cell walls avoiding their permeabilization and then the water is removed by action of alcohol.

Biofilm samples, in duplicate, were first washed carefully with a sterile saline solution (0.85 % NaCl) and covered for 12 hours in 1 mL of glutaraldehyde solution 3% wt. in sodium cacodylate buffer (see composition in Table A.9). Then biofilms were dehydrated with solutions of increasing ethanol concentration (10, 25, 50, 75, 90 and 100 %) for 15 min in each. The next steps of CO₂-drying and Au-coating were similar to those described above for the materials as were the observation conditions of SEM-EDS.

2.5 Microbiological Methods

2.5.1 Total Cell Numbers

The enumeration of the total cells in water samples and in diluted biofilm suspensions was performed with a blue fluorescent nucleic acid stain (DAPI, 4',6-diamidino-2-phenylindole, Sigma D9542). DAPI binds preferentially to double-stranded DNA by forming a stable complex which fluoresces about 20 times more than unbound.

The staining method was described elsewhere (Brunk *et al.*, 1979). DAPI working solution was 100 µg/mL, bacterial-free by filtration through by 0.2 µm membrane and stored in dark at 4° C. Water or suspended biofilm samples were gently homogenized in vortex for 1-2 min and a small volume (0.5 to 4 mL) was filtered through a 0.22 µm black polycarbonate membrane (WHAT110656). When bacteria were aggregated, a surfactant X-100 [Triton 0.001 % wt.] was added to disperse the cells. After 10 min of incubation with 200 - 500 µL of DAPI solution in the dark, the polycarbonate membrane was washed with and mounted with nonfluorescent immersion oil (Merck 1.04699) on glass microscope slides.

The membranes with the bacteria stained with DAPI (excitation = 365 nm; emission = 420 nm) were examined using an EF microscope with 1000x magnification. In each viewing field, 10 to 50 cells should be counted. A minimum of 300 cells or a sufficient number of microscopic fields were counted in order to obtain a coefficient of variation of less than 30 %.

The average and the standard deviation of the density of liquid samples is determined by the following equation

$$\text{Total cells/mL} = \frac{(\bar{x} \pm s) A_{\text{memb}} d}{A_{\text{field}} V_{\text{filt}}} \quad (2.12)$$

in which $\bar{x}$ and s are the average and the standard deviation of the number of cells counted (cell), A_{field} is the area of the viewing field (grid = $1 \times 10^{-4} \text{ cm}^2$ under $\times 1000$), A_{memb} is the membrane total area (2.27 cm^2), V_{filt} is the sample volume filtered through the membrane (mL) and d is the dilution factor. For the biofilm samples, the following equation should be used:

$$\text{Total cells/cm}^2 = \frac{(\bar{x} \pm s) A_{\text{memb}} d V_{\text{susp}}}{A_{\text{field}} V_{\text{filt}} A_{\text{biof}}} \quad (2.13)$$

in which A_{biof} is the coupon area with the biofilm that was suspended (cm^2) and V_{susp} is the total suspension volume (10 - 25 mL).

2.5.2 Metabolic Active Cell Numbers

The redox dye CTC (5-cyano-2,3-ditolyl tetrazolium chloride) can be employed for direct epifluorescent microscopic counting of metabolically active bacteria (Rodriguez *et al.*, 1992; Schaule *et al.*, 1993) in dispersed biofilm and in water

samples. Oxidized CTC is nearly colorless and nonfluorescent; however, the compound is readily reduced via electron transport activity to fluorescent, insoluble CTC-formazan, which accumulates intracellularly.

The CTC stock solution was prepared at 50 mM, was filtrated through a 0.22 μm membrane and stored in dark and at 4 °C.

The samples were incubated with 4 mM CTC solution and 9 % R₂B medium (with the same composition of R₂A without agar, in section A.1) for 4 hours in the dark at 28 °C and 200 rpm. Then, the stained suspension was filtered through a 0.22 μm black polycarbonate membrane and the metabolic active bacteria were examined using the EF microscope with filter cube N2.1 since CTC excitation and emission occurred at 450 and 630 nm, respectively. The enumeration of cells and calculus formulas was performed as described above (section 3.4.2).

2.5.3 Cultivable Cell Numbers

The heterotrophic plate count is a procedure for estimating the number of colony forming units which corresponds to the cultivable bacteria. The agar medium used was the R₂A (Reasoner and Geldreich, 1985) (see composition in section A.1) that was widely tested and accepted as the best nutritive medium to cultivate bacteria in drinking water systems (Korsholm and Sogaard, 1987; Gibbs and Hayes, 1988; Uhl and Schaule, 2004). The cultivable bacteria counts were estimated by the spread plating method using 100 μL of samples or dilutions thereof from dispersed biofilm and water samples. The cultures were incubated for 7 days at 22°C. The counted plates contained between 30 and 300 colony forming units.

The average of the density of liquid samples is determined by the following equation

$$\text{Total cells/mL} = \frac{\bar{x} + s}{10^{-d} V_{\text{add}}} \quad (2.14)$$

in which $\bar{x}$ and s are the average and the standard deviation of the number of cells that form colonies (cell), V_{add} is the sample volume added to the agar plate (0.100 mL) and d is the dilution factor.

For the biofilm samples, the following equation should be used:

$$\text{Total cells/cm}^2 = \frac{(\bar{x} + s) V_{\text{susp}}}{10^{-d} V_{\text{add}} A_{\text{biof}}} \quad (2.15)$$

in which A_{biof} is the coupon area with the biofilm that was suspended (cm^2) and V_{susp} is the total suspension volume (10 - 25 mL).

2.5.4 Fluorescence *in situ* hybridization (FISH)

Fluorescence *in situ* hybridization is a diagnostic technique to identify directly bacteria in mixed and complex samples in their natural environment, without cultivation. This technique is based in the detection of nucleic acid sequences with a fluorescently labeled oligonucleotides probe that hybridizes specifically to its complementary target sequence within the intact bacteria (Moter and Gobel, 2000). FISH analysis was performed as described previously (Manz *et al.*, 1992; Amann *et al.*, 1995) with some modifications. Low levels of metabolic activity can cause low signal intensity after *in situ* hybridization, which may be overcome by probe active counts. This technique, firstly proposed by Kogure *et al.* (1979) and later modified by Kalmbach *et al.* (1997b), relies on the incubation of bacteria in nutritive medium supplemented with a DNA-gyrase inhibitor that will increase DNA content whilst at the same time preventing cell division. This procedure may be called direct viable counts (DVC) when combined with fluorochromes or probe active counts (PAC) when coupled to FISH probes.

Several probes were used for the specific detection of bacterial groups accordingly to what was expected to be present in the drinking water biofilm and reactor water samples. The phylogenetic groups of targeted bacteria and the probe characteristics (specificity, coverage and target position in the 16S or 23S rRNA) are indicated in Figure 2.5 and Table 2.6.

The coverage indicates the percentage of sequences within the target group which show full match to the probe sequence, as determined by using the RDP probe match tool (Ribosomal Database Project v9.41).

Bacteria were discriminated partially and separated in groups belonging to the phyla *Bacteroidetes*, *Proteobacteria*, *Actinobacteria* and *Nitrospira*.

Three probe targeting *Alpha*-, *Beta*- and *Gamma*-subclasses of *Proteobacteria* were used (ALF968, BET42a and GAM42a). Bacteria able to oxidize ammonia to nitrite (AOB) and belonging to *Betaproteobacteria* were targeted with probe Nso190. In

the phylum *Nitrospira*, only the nitrite oxidizing bacteria (NOB) belonging to *Nitrospirales* were scanned with the Ntspa712.

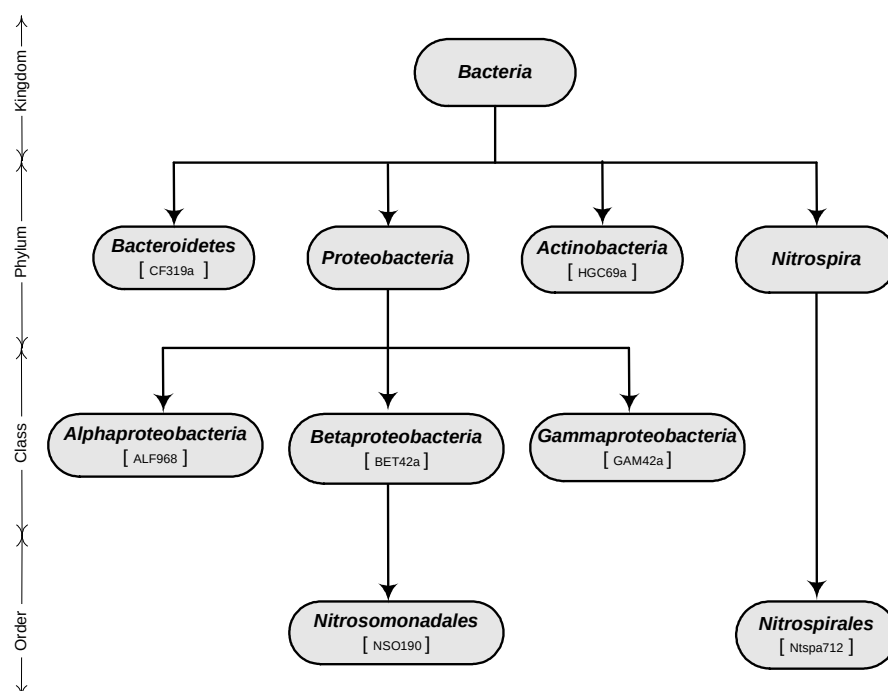


Figure 2.5 - The bacterial phylogenetic groups targeted by FISH with the corresponding used probes indicated in square brackets.

Table 2.6 - Specificity and target position of each oligonucleotides probes.

Oligonucleo- tides probes	Specificity	Coverage ¹ (%)	Target position ²
ALF968	<i>Alphaproteobacteria</i> except Rickettsiales	68	16S rRNA, 968-985
BET42a	<i>Betaproteobacteria</i>	n.d.	23S rRNA, 1027-1043
GAM42a	<i>Gammaproteobacteria</i>	n.d.	23S rRNA, 1027-1043
CF319a	<i>Bacteroidetes</i> (most <i>Flavobacteria</i> , some <i>Bacteroidetes</i> , some <i>Sphingobacteria</i>)	38	16S rRNA, 319-336
HGC69a	<i>Actinobacteria</i> (high G+C Gram- positive bacteria)	n.d.	23S rRNA, 1901-1918
Nso190	<i>Betaproteobacterial</i> ammonia- oxidizing bacteria	n.d.	16S rRNA, 189 - 207
Ntspa712	most members of the phylum <i>Nitrospira</i>	56	16S rRNA, 712 - 732

¹ The percentage of sequences within the target phylogenetic group which show a full match. ² Position in 16S rRNA of *Escherichia coli* strain K12.

The 5'-ends of the oligonucleotides (except for the competitor probes) were labeled with indocarbocyanine (Cy3; excitation = 550 nm; emission = 570 nm) or with fluorescein-carboxamid (Fluos or FAM; excitation = 490 nm; emission = 520 nm). The oligonucleotides were purchased from Thermo Company. 10x concentrated stock solutions were prepared (300 ng/μL for probes with Cy3 and 500 ng/μL for probes with Fluos) in ultrapure water and kept at -20 °C until prior use. Although Fluos-labeled probes permitted to visualize the stained bacteria, DAPI emission interfered with the Fluos emission when exposed for longer periods (during the bacteria counting). For this reason, all FISH counting were performed with the probes labeled with the Cy3.

Non-fluorescent competitor probes were used in an equal amount with probes BET42a, GAM42a and Ntspa712 to obtain optimal stringency conditions. BET42a and GAM42a served as competitors for each other.

Hybridization must be carried out under stringent conditions for proper annealing of the probe to the target sequence. The DNA sequences of each probe as well as the corresponding stringencies in the hybridization buffer (concentration of formamide and NaCl) are indicated in Table 2.7. The oligonucleotide probes and the FISH technique were tested and controlled every time was performed the hybridization by simultaneously applying positive and negative controls of each probe (Table 2.8).

Table 2.7 - Targeted DNA sequences and formamide stringencies in the hybridization buffer applied for each probe.

Oligonucleotides probes	Sequence (5' -> 3')	FA ² (%)	Reference
ALF968	GGT AAG GTT CTG CGC GTT	20	(Neef, 1997)
BET42a	GCC TTC CCA CTT CGT TT	35	(Manz <i>et al.</i> , 1992)
GAM42a	GCC TTC CCA CAT CGT TT	35	(Manz <i>et al.</i> , 1992)
CF319a	TGG TCC GTG TCT CAG TAC	35	(Manz <i>et al.</i> , 1996)
HGC69a	TAT AGT TAC CAC CGC CGT	25	(Roller <i>et al.</i> , 1994, 1995)
Nso190	CGA TCC CCT GCT TTT CTC C	55	(Mobarri <i>et al.</i> , 1996)
Ntspa712	CGC CTT CGC CAC CGG CCT TCC	50	(Daims <i>et al.</i> , 2001)

²Formamide concentrations in the hybridization buffer.

Table 2.8 Organisms used as positive and negative controls for each probe.

Oligonucleotides probes	Positives controls	Negatives controls
ALF968	<i>Sphingomonas paucimobilis</i>	<i>Ralstonia basilensis</i>
BET42a	<i>Ralstonia basilensis</i>	<i>Sphingomonas paucimobilis</i>
GAM42a	<i>Pseudomonas chlororaphis</i>	<i>Ralstonia basilensis</i>
CF319a	<i>Flavobacterium aquatile</i>	<i>Ralstonia basilensis</i>
HGC69a	<i>Gulosibacter molinativorax</i>	<i>Ralstonia basilensis</i>
Nso190	<i>Nitrosomonas europea</i>	<i>Ralstonia basilensis</i>
Ntspa712	<i>Leptospirillum ferrooxidans</i>	<i>Ralstonia basilensis</i>

FISH procedure included the following steps:

- (i) bacteria sampling - in order to have enough bacteria in an homogenized suspension;
- (ii) sample incubation - in a nutritive medium supplemented with the pipemidic acid antibiotic;
- (iii) fixation - cell permeabilization for penetration of probes and dehydration;
- (iv) immobilization - in a 10 well plate;
- (v) hybridization – with the probes for detecting with the respective target sequences;
- (vi) washing – to remove unbound probes;
- (vii) EF microscope visualization and counting.

Sample preparation. Water samples with 100 to 1500 mL were concentrated by successive steps of filtration through 0.22 µm polycarbonate membranes (WHATMAN 11114) and centrifugation at 14000 rpm. The pellet was re-suspended in 1 mL of 1x PBS (composition in Table A.2) sonicated with the sonicator Vibra Cell (SONICS model VCX 130) with 3 mm-diameter probe (Ref. number 630-0422). Biofilm samples were removed from the coupons, suspended in 10 mL of 1x PBS and then concentrated as described above.

Sample incubation. The concentrated samples were incubated with $0.5 \times R_2B$ amended with 30 mg/L of pipemidic acid at 30 °C and 120 rpm overnight. Tests were performed to confirm that total bacteria number was maintained constant. Samples were after concentrated to a final volume of 100 µL.

Cell fixation. 300 µL of paraformaldehyde solution [4 % wt., SIGMA P6148] was added and the mixture was incubated at 4 °C for 3 h. The samples were then

centrifuged and washed twice with 1×PBS. The pellet was re-suspended in 200 µL of PBS + 200 µL of cold ethanol and kept at -20 °C until used.

Immobilization. 5 - 10 µL of sample was immobilized on 10-well glass slides with 7 mm of diameter printed with black epoxy resin (Marienfeld, ref 2423). While samples were drying at 46 °C, fresh hybridization and washing buffers were prepared and the latter was placed in the oven at 46 °C (see buffers compositions in Table A.3 and Table A.4). Then samples were dehydrated with ethanol (50, 80 and 100 %; 3 min each) and dried. When HGC69a probe was used, 20 µL of lysozyme (SIGMA L-6876) solution (10 mg/mL) were added for 15 min and then, ethanol dehydration and drying steps were repeated.

Hybridization. In each well, 8 µL hybridization buffer and 2 µL of fluorescent probe were added and the plates were incubated at 46 °C for 3 h in an equilibrated dark and humid chamber. If two probes were used simultaneously 6 µL hybridization buffer and 2 µL of each probe were added to each well.

Washing. The well plate was washed with pre-warmed (48 °C) washing solution to remove the major amount of hybridization buffer and probe. After adding 15 µL of DAPI (100 µg/mL) to the washing buffer containing the well plate, the samples were incubated for 15 min at 48 °C in dark. Subsequently, the well plates were gently washed in sterilized water and dried.

EF microscope visualization and counting. Before visualization of samples in EF microscope, the wells were covered with mounting oil (Merck 1.04699) and a coverslip (Marienfeld, 24x50 mm, n°1-8590). In each viewing field, DAPI-stained cells and probe-specific cells were counted and the mean and standard deviation of the percentage of cells visualized by probe-specific cells were calculated. Samples were analyzed in duplicate.

2.5.5 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is an electrophoretic technique that separates double-stranded DNA fragments of the same size that differ in base sequence (Rochelle, 2003). A gradient of DNA denaturant is used to “melt” a double-stranded DNA fragment moving across the gel, through which stops migration. The differences in melting properties are controlled largely by differences in base sequences. Therefore, each band observed on the gel, represents a specific sequence of a gene fragment that may vary by as little as one nucleotide in their sequences (Rochelle, 2003). When using

16S rDNA fragments, this method gives a detailed picture of the number of phylotypes (distinct 16S rRNA genes) present in a sample. A scheme of the complete procedure is presented in Figure 2.6.

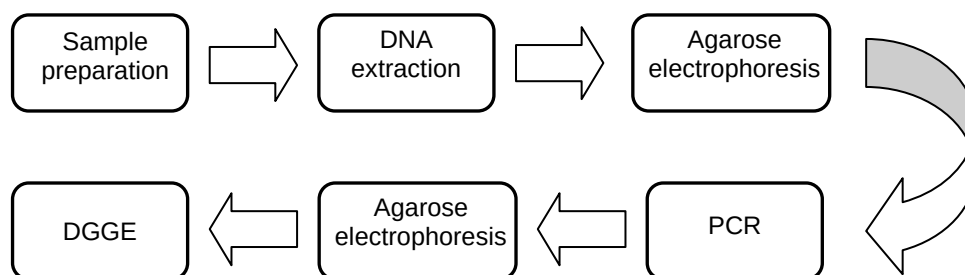


Figure 2.6 - Scheme of the complete procedure to perform DGGE analysis.

Total DNA was extracted from biofilm and water samples and amplified using PCR primers for eubacterial 16S rDNA. Total DNA and PCR products were visualized by agarose gel electrophoresis in order to check their presence and size. Finally, PCR products were separated by DGGE. When the amount of the extracted DNA was not enough to obtain a band profile in the DGGE gel, a nested-PCR was performed according a procedure described elsewhere (Hoefel *et al.*, 2005b). Nested-PCR is composed of two amplifications performed sequentially: in the first PCR the complete 16S rDNA is amplified (1465 bp) and in the second one, the primers allow a replication of fragments of 180 bp.

Sample preparation. The biofilm suspensions (10 - 25 mL) and water samples (1L-20L) were filtered through 0.22 μ m polycarbonate sterile filters. Cells were removed from the filters with a 1 mL tip and TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and harvested by centrifugation (14000 rpm). The cultivable bacteria colonies were suspended in TE buffer in order to extract DNA for DGGE. To extract DNA from pure bacteria cultivated in nutritive agar plates, 2-3 colonies were collected to 1 mL sterile tube. These pellets could be frozen at -20 °C for several weeks prior to DNA extraction.

DNA extraction. Total DNA extraction was performed using The Genomic DNA Purification kit (MBI Fermentas K0512), according to the manufacturer's instructions, with some modifications (Henriques *et al.*, 2004). The bacterial pellets were resuspended in 200 μ L of TE buffer containing 10 mg/mL of lisozyme and incubated for 2 h at 37 °C. 400 μ L of lysis solution were added and the mixture was incubated at 65 °C for 10 min. Immediately 600 μ L of chloroform was added and after gently emulsification/inversion, the samples were centrifuged for 15 min. The

upper aqueous phase containing the DNA was transferred to a new tube containing 720 μL of sterile deionized water with 80 μL of supplied 10 $\times$ concentrated precipitation solution and incubated at 4 $^{\circ}\text{C}$ for 30 min. After centrifugation for 20 min, the supernatant was removed and the pellet was dissolved in 100 μL of 1.2 M NaCl solution by gentle vortexing. Then 300 μL of cold ethanol was added, the DNA was let for precipitation at -20 $^{\circ}\text{C}$ for 30 min and spun down for 20 min. The ethanol was removed; the pellet was dried at room temperature overnight and dissolved in 50-100 μL of sterile MilliQ water.

Electrophoresis of total DNA in 1% agarose gel. The extracted total DNA of each sample was checked by electrophoresis in 1 % agarose gel at 90 V for 1.5 h in 1 $\times$ TAE buffer [40 mM Tris (hydroxymethyl) aminomethano, 20 mM Acetic acid and 1 mM EDTA buffer (pH 8.0)]. The gel was prepared by heating and mixing until clarification followed by 30 min of polymerization. The gel was placed in the electrophoresis tank filled with 1 $\times$ TAE buffer and 5 μL of total DNA mixed with 2 μL of loading dye (40% sucrose, 0.25% Bromophenol blue, and 0.25% Xylene cyanol) were added to each well. The size of the DNA band was checked against a calibrated DNA ladder (GeneRulerTM DNA Ladder Mix, MBI Fermentas). The gel was stained with 15 $\mu\text{g/L}$ ethidium bromide, visualized on a UV transilluminator and the gel image was digitalized with a photographic camera.

Polymerase Chain Reaction (PCR). A portion of the 16S rDNA gene from most bacteria was amplified using the primers 518r (5'-ATT ACC GCG GCT GCT GG-3') and 338f-GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG-3'), the GC clamp is the underlined portion (Muyzer *et al.*, 1993). The primers were synthesized by Thermo Scientific. PCR reactions were performed with recombinant Taq polymerase, nucleotides and buffers from MBI Fermentas (EP0404, R0192), in a thermocycler (MyThermalCycler, Biorad). All reactants were kept at -20 $^{\circ}\text{C}$ and divided in aliquots of appropriate volume to avoid contaminations and deterioration due to defreezing/freezing when used. The water used was of high purity, MilliQ after being filtrated, autoclaved twice and aliquoted in 1 mL in sterile tubes and kept at -20 $^{\circ}\text{C}$. The reactant aliquots and the DNA samples were kept in ice when the reaction mixtures were prepared together according to the volumes indicated in Table A.5. This mixture was then divided in 49 μL in each Eppendorf tube to which 1 μL of DNA template was added after. Each 50 μL reaction mixture was composed of 3 mM of MgCl_2 , 1 $\times$ Taq buffer, 5 % (v/v) of dimethylsulfoxide

(DMSO), 200 μ M of each nucleotide, 15 pmoles of each primer, 1 unit of *Taq* DNA polymerase and 1 μ L of DNA template. The reaction tubes were placed in the thermocycler that was turned on to start the temperature program previously inserted. After an initial denaturation step at 94 °C for 5 min, amplification continued for 30 cycles with denaturation at 92 °C for 30 s, annealing at 55°C for 30s and extension at 72 °C for 30 min. A final extension step at 72 °C for 7 min was included. Negative control reactions without a template DNA were carried simultaneously.

Nested Polymerase Chain Reaction. Nested-PCR is composed of 2 sequential PCR cycles and was performed when the extracted total DNA was not enough to perform just one PCR cycle (as described above) and obtain bands in the DGGE gel. In this case, total 16S rRNA gene was amplified using the 27f (5'-GAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACCTTGTTACGACTT-3') primers and then a 2nd PCR cycle was performed using the 1st PCR products as DNA template and the 338f-GC and 518r primers referred above.

In the 1st PCR cycle, the reaction mixtures were prepared according the volumes indicated in Table A.6, divided in 22.5 μ L in each tube and to which 2.5 μ L of DNA template was added. Each 25 μ L of reaction mixture was composed of 3 mM of MgCl₂, 1 $\times$ *Taq* buffer, 5 % (v/v) of dimethylsulfoxide (DMSO), 200 μ M of each nucleotide, 25 pmoles of each primer, 1.25 units of *Taq* DNA polymerase and 2.5 μ L of DNA template. The reaction mixtures were subjected to the following temperature program. An initial denaturation step at 95 °C for 10 min was carried out; followed by 30 cycles of 30 s at 94 °C (denaturation), 1 min at 50 °C (annealing) and 2 min at 72 °C (extension). A final extension step at 72 °C for 10 min was included. Negative control reactions without any template DNA were carried out simultaneously.

In the 2nd PCR cycle, the primers were 518r and 338f-GC clamp and the reaction volume was 50 μ L in which 3 μ L were the DNA template that was the final product obtained in the 1st PCR. The reaction mixtures had the same composition used in the 1st PCR (Table A.6) and were subjected to the following temperature program: After an initial denaturation step at 95 °C for 10 min, amplification started with 4 cycles in denaturation at 94 °C for 1min, annealing starting at 65°C for 1 min, and extension at 72 °C for 2 min. After every four subsequent cycles the annealing temperature was reduced by 2 °C until 55°C at which point the cycle was repeated 11 more times followed by a final extension step at 72°C for 10 min.

Electrophoresis of PCR products in 1.0 % agarose gel. PCR products of 180 bp long were checked by electrophoresis in 1.0 % agarose gels at 90 V for 40 min in TAE buffer. 5-8 μ L of PCR product and 2 μ L of loading dye were added to each well. The size of the DNA band was checked against a calibrated DNA ladder of 100-5000 bp (GeneRuler™ DNA Ladder Mix, MBI Fermentas). The gels were stained, visualized and the gel image was digitalized as described above.

Denaturing gradient gel electrophoresis. The DGGE was performed in a DCode™ system (Bio-Rad Laboratories) using 1 mm vertical gel with 8 % wt. polyacrylamide (acrylamide:bisacrylamide 37.5:1), 1 $\times$ TAE and a 35 - 60 % denaturing vertical gradient formed with urea and formamide. 100 % denaturing gradient corresponds to 7 M urea and 40 % (v/v) deionized formamide. The 35 % and 60 % gels were prepared separately (see composition in Table A.7), mixed in the gel gradient delivery (Bio-Rad model 475) and polymerized at room temperature overnight. The polymerization agents were 14 μ L TEMED (tetramethylethylenediamine, Biorad) and 140 μ L of 10 % wt. ammonium persulfate (Biorad). The gel was mounted and placed inside the electrophoresis tank which was filled with 7 L of 1 $\times$ TAE buffer pre-warmed at 60 °C.

PCR products (20 - 35 μ L) were mixed with 5 μ L of loading dye and injected in the gel wells. Electrophoresis was performed initially at 60 °C and 20 V for 15 min and then followed by a 5.5 h-long step at 100 V and 60 °C. The gel was stained for 15 min with ethidium bromide and visualized on a UV transilluminator. The gel image was digitalized with a photographic camera.

Band analysis. Band analysis was performed to compare different samples in the same DGGE gel by matching the bands in the different lanes. This clustering analysis is described in section 2.7.

2.5.6 Gram Staining

Bacteria can be differentiated in two groups according to their cell walls using the Gram staining in which the ones with the thickest walls stain in purple (Gram-positive bacteria) and the others in pink (Gram-negative bacteria) (Beveridge, 2001).

The bacteria preparation was performed on a slide from a fresh and pure culture that was fixed by the flame. Then the stains were added sequentially as follows. Firstly, the crystal violet stain (10 % wt. in ethanol at 95 %) was added to the preparation

and washed 30 seconds after. Secondly, Gram's iodine (3.3 g iodine/L + 6.7 g KI/L) was added and after 30 seconds, the slide is washed. Thirdly, the decolorizing solution (80 % ethanol + 20 % acetone) was applied while color was being washed away. Finally, the safranin (0.25 % wt. in ethanol at 95 %) was added and the stain in excess was washed 30 seconds after. After air drying and oil mounting, the preparation can be viewed in the light microscopy.

2.5.7 API 20 NE Identification

Microbial composition of the cultivable Gram-negative fraction of water samples was identified with a kit for the non-enteric and non-fermenting gram-negative rods (API 20NE, Biomerieux). This phenotypic identification may be assigned to the species or genus levels according to substrate assimilations. The protocol was performed according to the manufacturer instructions.

Some Gram-negative non-fermenters such as *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Stenotrophomonas maltophilia*, and *Alcaligenes* spp. are considered opportunistic bacteria that are ubiquitous in the environment and may cause infections in immunocompromised people (Berthelot *et al.*, 2005; Bosshard *et al.*, 2006; Paterson, 2006).

2.5.8 Detection of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic pathogen that was detected in biofilms and in suspension of several drinking water systems (de Victorica and Galvan, 2001; Penna *et al.*, 2002; Kilb *et al.*, 2003; Emtiazi *et al.*, 2004; Lee *et al.*, 2006; September *et al.*, 2007).

One liter of water samples was collected in a sterile flask and filtered through a 0.2 µm polycarbonate membrane (WHATMAN 111106). Then the filter was placed on the selective cetrимide / nalidixic acid agar medium. The *Pseudomonas aeruginosa* selective medium was composed by 44.5 g/L of cetrимide agar (Merck), 10 mL/L of glycerol and 15 µg/L of nalidixic acid (Merck). The cultures were incubated for 2 days at 36° C and the membrane filters were checked for growth after 24 h and 48 h (DIN EN 12780 2002). All grown colonies with a blue-green pigmentation are considered confirmed *Pseudomonas aeruginosa*. All colonies not showing this pigmentation but fluorescing under UV light are suspected *P. aeruginosa* and must be confirmed with acetamide solution. The reddish-brown pigmentating colonies

are suspected *P. aeruginosa* and confirmation is necessary with oxidase test, acetamide solution and king's B medium.

2.6 Mass Balances and Cell Number Kinetics

The cell growth rates in biofilm and bulk water was determined by counting the cell total numbers obtained from bulk water, tap water and biofilm samples.

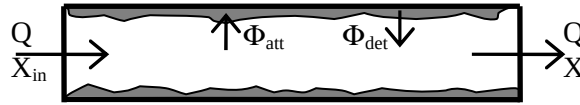


Figure 2.7 - Bulk water cell balance in the reactor system. (Φ_{att} - rate of cell attachment; Φ_{det} - rate of cell detachment from the surface; Q - water flow rate; X_{in} - inlet water cell density; X - outlet water cell density (= reactor bulk water cell density, assuming a perfectly mixed system).

Consider the cell balance in the reactor represented in Figure 3.5. The accumulation of cells in bulk is related to the net transport through the inlet and outlet, the cell removal and attachment to the biofilm, as well as to the bulk phase net growth, and it can be expressed by the following equation:

$$V \frac{dX}{dt} = Q(X_{in} - X) + \mu_{bulk} VX - \Phi_{att} + \Phi_{det} \quad (2.16)$$

where V is the volume of the system (mL), Q is the inlet flow rate (mL/day), X_{in} and X are the suspended cell densities (cell/mL) in the inlet and outlet water, respectively, X_b is the biofilm cell number per unit surface area (cell/cm²), Φ_{det} is the biofilm removal rate (cells/day), Φ_{att} is the rate of cell attachment to the surface (cells/day) and μ_{bulk} is the specific cell growth rate in suspension (day⁻¹). The cell balance related to the biofilm phase is expressed by:

$$A \frac{dX_b}{dt} = \Phi_p - \Phi_{det} \quad (2.17)$$

where A is the internal surface area (cm²) and Φ_p is the biofilm production rate (cells/day) in the biofilm. Eq. (2.17) describes biofilm development over time as the result of the competition between a biomass production process (which includes attachment of new cells and cell growth within the biofilm) and a biomass removal

process (whereby the rate of cells removed from the biofilm to the bulk water increases with the number of cells already attached):

$$\Phi_p = \mu_{\text{biofilm}} X_b A + \Phi_{\text{att}} \quad (2.18)$$

$$\Phi_{\text{det}} = \beta X_b A \quad (2.19)$$

where μ_{biofilm} is the specific cell growth rate inside the biofilm (day^{-1}) and $1/\beta$ represents the average residence time of cells within the biofilm.

An overall biofilm model can then be established considering the following cell number balance in the biofilm over time (Characklis and Marshall, 1990; Melo and Bott, 1997; Melo and Vieira, 1999):

$$A \frac{dX_b}{dt} = \Phi_p - \beta X_b A \quad (2.20)$$

Considering the assumption that $\Phi_p = \text{constant}$, which was demonstrated by several authors (Melo and Vieira, 1999; Pereira *et al.*, 2002b), the integration of Eq. (2.17) leads to:

$$X_b = (X_b)_{\text{max}} (1 - e^{-\beta t}) \quad (2.21)$$

and

$$(X_b)_{\text{max}} = \frac{\Phi_p}{A\beta} \quad (2.22)$$

where $(X_b)_{\text{max}}$ is the steady-state biofilm cell number per unit surface area. Similar asymptotic models have been shown to describe biofilm dynamics over time by several authors (Belkhadir *et al.*, 1988; Capdeville *et al.*, 1988; Characklis and Marshall, 1990).

Although the number of cells in suspension might be important for the start up of biofilm formation on surfaces, cell attachment to the surface does not seem to affect biofilm development in a significant way after those initial moments, as confirmed experimentally (Bott and Miller, 1983; Melo and Vieira, 1999). Therefore, Eq. (2.22) can be simplified into:

$$\Phi_p = \mu_{\text{biofilm}} X_b A \quad (2.23)$$

Therefore, at steady-state, the (final) specific biofilm cell growth rate in the biofilm, μ_{biofilm}^* , is related to the final cell number per unit surface area, $(X_b)_{\text{max}}$, and the biofilm production rate, Φ_p , by the following equation:

$$\mu_{\text{biofilm}}^* = \frac{\Phi_p}{(X_b)_{\text{max}} A} \quad (2.24)$$

which means that cell growth in the biofilm in steady state is equal to the cell detachment from biofilm.

In order to determine μ_{bulk} , Eq. (2.16) and (2.17) were added and at steady state (the time derivatives are zero):

$$0 = Q(X_{\text{in}} - X^*) + \mu_{\text{bulk}}^* X^* V + \mu_{\text{biofilm}}^* (X_b)_{\text{max}} A \quad (2.25)$$

where X^* and $(X_b)_{\text{max}}$ are the steady state values of suspended cell density and the biofilm cell number per unit surface area, respectively. The cell growth rate in bulk in steady state may be obtained once μ_{biofilm}^* is known.

$$\mu_{\text{bulk}}^* = \frac{Q(X^* - X_{\text{in}}) - \mu_{\text{biofilm}}^* (X_b)_{\text{max}} A}{X^* V} \quad (2.26)$$

Defining an overall specific cell growth rate, μ_{overall} , as a weighed average of the cell growth rates in the biofilm and in the bulk water (Boe-Hansen *et al.*, 2002a), then

$$\mu_{\text{overall}} (XV + X_b A) = \mu_{\text{bulk}}^* XV + \mu_{\text{biofilm}}^* X_b A \quad (2.27)$$

Therefore, taking into account Eq. (2.26), the steady state overall specific rate growth can also be given by:

$$\mu_{\text{overall}}^* = \frac{Q(X^* - X_{\text{in}})}{X^* V + (X_b)_{\text{max}} A} \quad (2.28)$$

2.7 Statistical Analysis

The statistical analyses used in the present work are summarized above.

Propagation of random errors in calculus. The data obtained experimentally was averaged ($\bar{x}_i$) and the standard deviations (s_i) were obtained. When performing mathematical calculus using this data ($x_1, x_2, \dots$), the final value (y) has associated also a standard deviation according to:

$$s_y = \left| s_{x_1} \frac{\partial y}{\partial x_1} \right| + \left| s_{x_2} \frac{\partial y}{\partial x_2} \right| + \dots \quad (2.29)$$

Comparison of experimental means. Considering two independent samples (A and B), the two-sided t-test (without the assumption of equal standard deviations) was performed according to:

$$t = \frac{\bar{x}_a - \bar{x}_b}{\sqrt{\frac{s_a^2}{n_a} + \frac{s_b^2}{n_b}}} \quad (2.30)$$

$$\text{and degrees of freedom} = \text{INT} \left[\frac{\left(\frac{s_a^2}{n_a} + \frac{s_b^2}{n_b} \right)^2}{\frac{s_a^4}{n_a^2(n_a - 1)} + \frac{s_b^4}{n_b^2(n_b - 1)}} \right] \quad (2.31)$$

Correlation and the corresponding significance. Correlation (r) between two experimental variables was evaluated by the product-moment coefficient:

$$r = \frac{\sum_i [(x_i - \bar{x})(y_i - \bar{y})]}{\sqrt{\left[\sum_i (x_i - \bar{x})^2 \right] \left[\sum_i (y_i - \bar{y})^2 \right]}} \quad (2.32)$$

and the corresponding significance was obtained by two-sided t-test for 5 % and $(n-2)$ degrees of freedom.

The statistic test is performed by:

$$t = \frac{|r| \sqrt{n-2}}{\sqrt{1-r^2}} \quad (2.33)$$

Detection limits. The detection limits may be described as the measured value which corresponds to an instrumental signal that is significantly different from the

“blank” or the “background”. According to Miller and Miller (2000), the limit of detection may be determined from the calibration data, being defined by

$$\text{Limit of detection} = y_b + 3 s_{y/x} \quad (2.34)$$

and

$$s_{y/x} = \sqrt{\frac{\sum_i \left(y_i - \hat{y}_i \right)^2}{n - 2}} \quad (2.35)$$

in which y_b is the “blank” signal that corresponds to the intercept obtained from the calibration curve, $s_{y/x}$ is the overall random errors in the y-direction, $\hat{y}_i$ are the estimated values and $n-2$ the number of degrees of freedom.

Errors of parameters from regression linear analysis. The standard errors associated to the intercept (s_a) and the slope (s_b) obtained from regression line are defined as follows:

$$s_a = s_{y/x} \sqrt{\frac{\sum_i X_i^2}{n \sum_i (X_i - \bar{X})^2}} \quad s_b = \frac{s_{y/x}}{\sqrt{\sum_i (X_i - \bar{X})^2}} \quad (2.36)$$

Principal component analysis. This technique (PCA) is a multivariate technique that reduces the complexity of the data by decreasing the number of variables that need to be considered. Full details are given elsewhere (Landau and Everitt, 2004). The new derived variables (called the principal components) should explain at least from 70 to 90 % of the total variance of the data system. When the original variables are on very different scales, standardization should be performed before PCA, or the variables with larger variances will have higher influence in the analysis.

The principal components $y_1, y_2, \dots, y_n$ are defined as linear combinations of the original variables $y_1, y_2, \dots, y_n$ and are obtained as follows:

$$\begin{aligned}
y_1 &= A_{11}x_1 + A_{12}x_2 + \dots + A_{1n}x_n \\
y_2 &= A_{21}x_1 + A_{22}x_2 + \dots + A_{2n}x_n \\
&\vdots \\
y_n &= A_{n1}x_1 + A_{n2}x_2 + \dots + A_{nn}x_n
\end{aligned} \tag{2.37}$$

where the coefficients A_{ij} are calculated maximizing the amount of variance of the data system.

When performing the PCA analysis, the correlation matrix of the original variable is firstly determined and then the principal components are derived. This matrix shows also if the original variables are correlated or not by comparing the absolute correlation coefficient $|r|$ with $|r_{crit}|$. This critic value was obtained from two-sided t-test with a significance level of 5%.

The principal components are plotted for the variables and for the objects, allowing the interpretation of similarities between the objects by explaining with the variables. Principal component analysis was performed in SPSS (v 15.0, UTHSCSA).

Hierarchical Cluster Analysis. With this analysis, the data was represented in dendrograms that summarizes hierarchically the similarities between samples. In hierarchical methods, the members of inferior-ranking clusters (the most similar samples) become members of larger and higher-ranking clusters. In this thesis, the cluster technique was used to compare the band profiles of samples from the DGGE gel by matching the bands in the different lanes and grouping according the similarities. First, a binary matrix of the lane profile was built (1 = band presence, 0 = band absence) that was used to determine the similarity parameter between each two samples. This parameter was quantified according to Dice coefficient (or Czekanowski or Sorenson coefficient) in which joint absences are excluded from computation and matches are weighed double, defined as

$$Dice(X, Y) = \frac{2a}{2a + b + c} \tag{2.38}$$

where **a** is the number of presences found in both samples, **b** and **c** are the number of presences found only in each sample (**x** or **y**). With the similarity (or proximity)

matrix, a dendrogram was built by clustering the variables with the Unweighted Pair-Group Method using Arithmetic mean (UPGMA, also called Average Linkage Between Groups in SPSS). This statistical technique was performed in Excel and in SPSS (v 15.0, UTHSCSA).

3. Tap Water in the Distribution System

3.1 Introduction

Information from the drinking water monitoring was highly significant for the present study as it characterized the inlet flow of the reactors. The effects of temperature (according to the seasonal period), flow rate and long stagnancy periods on the tap water were also evaluated. This type of samples was also used to define the experimental conditions when the microbiological techniques (FISH and DGGE) were being implemented, as it generally presented low cell densities and high fraction of shrunk cells with lower metabolically activity and cultivability capacity (and thus low amounts of rDNA).

3.2 Experimental Methodology

In this chapter are presented all the results obtained in the analysis of the drinking water coming directly from the tap connected to the public drinking water distribution. The drinking water was analyzed every time an experiment was running to check the effect of some operating parameters (e. g. temperature and shear stress) on the water properties.

Physicochemical Characteristics of Tap water. Several water samples were collected to sterile bottles directly from the tap (**Exp. 3.1**). These samples were

collected during a period of 3 years (in winter and summer) from a tap that was regularly used. The physicochemical (namely, temperature, pH, total dissolved solids, inorganic carbon, total organic carbon, dissolved organic carbon, phosphorous, nitrogen and chlorine) were measured according to procedures described in section 2.3. The analyses were performed within 5 hours after collecting the samples, except for the carbon quantification: samples could be frozen for several weeks.

Microbiological Characteristics of Tap Water. The microbiological parameters (cells densities defined for the total, metabolically active and cultivable cells) were measured (**Exp. 3.2**) for several water samples coming from the tap, according to procedures described in section 2.4. These parameters were evaluated immediately after collecting the samples.

Effect of Stagnancy, Temperature and Flow. The effect of stagnancy was evaluated (**Exp. 3.3**) collecting 8 water samples of 1 L volume, continuously at 1.4 L/min, from a tap that had not been used for 1 month. The laboratory temperature was 28° C and, at least 5 m-long pipe was at the same temperature (external wall PVC pipe). Also, several tap water samples were collected during winter and summer and after periods of normal and continuously tap use (**Exp. 3.4**). Microbiological parameters were evaluated in terms of total, metabolically active and cultivable cells. With these results, information was obtained about the changes in the parameters with the temperature (seasonal period) and the flow rate.

Characterization of microbial populations. The overall microbial community composition was also studied by Fluorescent *in situ* Hybridization (FISH) and Denaturing Gradient Gel Electrophoresis (DGGE) techniques (sections 2.5.4 and 2.5.5) (**Exp. 3.5**). Several steps/conditions were tested such as the sample preparation, the extraction and the amplification of the bacterial DNA, and the sample incubation in a nutritive medium supplemented with the pipemidic acid antibiotic. When tap water samples were collected for DGGE studies by filtration and centrifugation, the pellets were frozen at -20 °C for several weeks before or after extracting the DNA. For FISH, the sample cells had to be immediately concentrated and fixated with formaldehyde and then were frozen at -20 °C for several months. For the cultivated fraction in R₂A (**Exp. 3.6**), each morphotype was purified through sub-culturing on the same media. Gram test was performed and then the non-enteric and non-fermenting gram-negative rods were identified with the kit API 20NE (Biomerieux). In **Exp. 3.7**, 16 samples of 1 L of tap water were collected to sterile flasks, filtered and the membranes were placed in the selective

cetrimide/nalidixic acid agar medium. This experiment was performed in order to evaluate if *Pseudomonas aeruginosa* was present in tap water.

3.3 Results and Discussion

3.3.1 Physicochemical Characteristics of Tap Water

The physicochemical parameters of all samples of tap water analyzed during the 3-years work are presented in Table 3.1 by the mean, the maximum and the minimum values. Generally these parameters were more or less constant within a certain range. However, it was observed higher temperatures in summer than in winter which promoted a slight increase of the organic carbon (especially in samples collected after long residence times). There was no significant effect of flow velocity on the physicochemical parameters.

The measured physicochemical parameters were in accordance with those allowed for waters for human consumption as defined by the following Portuguese Decrees - Law: no. 236/98, no. 243/201 and no. 306/07. The results presented in Table 3.1 indicated that Porto drinking water was practically neutral with low contents of dissolved solids, of organic carbon (that is all dissolved), of phosphorous, of nitrogen and of chlorine residuals.

3.3.2 Microbiological Parameters of Tap Water

Densities of total, metabolically active and cultivable cells were evaluated for several samples of water coming directly from the tap.

Total cell counting was performed after filtering the water sample and staining with DAPI. This blue fluorescent dye binds preferentially double-stranded DNA and forms strongly fluorescent DNA-DAPI complexes with high specificity (Brunk *et al.*, 1979; Kapuscinski and Szer, 1979). Although the fluorescence enhancement was described as proportional to DNA content, DAPI binds preferentially to AT clusters in the DNA minor groove (Kubista *et al.*, 1987) and intercalates the AU sites in RNA (Tanious *et al.*, 1992). Although DAPI stains other organism types, bacteria are easily distinguish by the different sizes and shapes.

The typical microscopic image of tap water bacteria (Figure 3.1a) shows them with small dimensions. The cells are not very bright probably due to the low amount of nucleic acids when driving under adverse life conditions (chlorine residuals, low

Table 3.1 - Measured physicochemical parameters of drinking water from the Porto distribution system and the recommended values (Exp. 3.1).

Parameters	Mean	Min	Max	Recommended Values ⁽¹⁾
T (°C)	15.9	11.1	16.9	12 - 25
pH	7.2	6.97	7.83	6.5 - 8.5
O ₂ (mg O ₂ /L)*	8.4	7.4	9.1	6.3 - 10.8
IC (mg C/L)	17.2	15.2	24.6	---
TOC (mg C/L)	2.73	1.85	3.45	---
DOC (mg C/L)*	2.12	1.35	2.67	---
k (µS/cm)	252	225	296	< 400
TDS (mg/L)	168	150	198	< 1500
Chlorine (mg Cl ₂ /L)*	0.18	0.12	0.31	---
Nitrate (mg NO ₃ ⁻ /L)	7.35	3.25	9.34	< 50
Nitrite (mg NO ₂ ⁻ /L)	0.012	< 0.01	0.014	< 0.1
Ammonia (mg NH ₃ /L)	< 0.1	---	---	< 0.5
Total nitrogen (mg N/L)	1.7	< 0.7	1.9	---
Total phosphorous (mg P/L)*	0.013	0.011	0.023	---

IC - inorganic carbon; TOC - total organic carbon; DOC - dissolved organic carbon; k - conductivity; TDS - total dissolved solids. All values are given as mean, minimum and maximum values. n = 39 (except * n = 10). (1) - Defined in the Portuguese Law. Some values are not indicated as research must be performed when abnormal changes occur.

temperatures, low nutrients content,...). The cells of these samples had to be counted quickly otherwise was completely photo bleached and the bacteria could no longer be distinguished from the background color. Other compounds (corrosion products, precipitates,...) were also stained but not counted as easily distinguished from bacteria due to the different shape and size. This non-specific-fluorescence was also observed by other author (Chang *et al.*, 2003).

The photo bleaching occurred when DAPI was exposed to the excitation light and the corresponding fluorescence faded due to an oxidative process of the fluorochrome by oxygen and triplet states (Gallardo-Escarate *et al.*, 2007) or chlorine residuals (Phe *et al.*, 2004). This process was retarded by using a special mounting medium with antifading reagents (Prolong Antifade Kit, Molecular Probes) that inhibit the generation and the diffusion of reactive oxygen. Divalent

cations and heavy metals may also reduce the fluorescence signal by quenching with the DAPI (Brunk *et al.*, 1979).

When these bacteria were in stagnant water for 1 month in the pipe immediately upstream of the tap, the DAPI picture showed them to be fatter and brighter (Figure 3.1b), which was attributed to an increase in cell growth at higher temperature. The size and the brightness may be also improved when drinking water bacteria was allowed to grow in a nutritive media (Figure 3.1c).

The 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) is a monotetrazolium redox dye which produces a fluorescent formazan when is reduced and precipitates entrapped inside the bacteria. CTC competes with the enzymes involved in the cellular activity (specifically with the dehydrogenase) acting as a reduced hydrogen acceptor. Thus, CTC is an indicator that intracellular redox reactions are occurring. This technique has been extensively used in different aerobic bacterial samples, including pure cultures (Rodriguez *et al.*, 1992; Strathmann *et al.*, 2000; Créach *et al.*, 2003), drinking water bacteria attached and suspended (Schaule *et al.*, 1993; Mcfeters *et al.*, 1995; Cook and Garland, 1997; Kalmbach *et al.*, 1997a; Schwartz *et al.*, 1998; McFeters *et al.*, 1999; Boe-Hansen *et al.*, 2003; Chang *et al.*, 2003), river samples (Servais *et al.*, 2001; Araya *et al.*, 2003; Freese *et al.*, 2006), groundwater and seawater (Rodriguez *et al.*, 1992; Schwartz *et al.*, 1998).

Metabolic activity comprises the reactions pathways to produce energy from nutrients that are degraded into smaller molecules (catabolism) or to synthesize components (lipids, proteins, polynucleotides, and polysaccharides) needed to build cells structures. Smith and McFeters (1997) demonstrated that CTC (redox potential -200 mV) was reduced by succinate or NADH dehydrogenases (redox potential -300 mV) when operating in a respiration chain. Several authors used the term “respiring activity” but dehydrogenases operate in other metabolic pathways, not necessarily the respiration (*e.g.*, glycolysis, ghosphogluconate pathway and the conversion of pyruvic acid into ethanol).

The metabolic activity was measured successfully in different facultative and obligate anaerobic bacteria (Bhupathiraju *et al.*, 1999) that accumulated reduced CTC intracellularly even without adding nutrients. Cultures of *E. coli* grown fermentatively or with fumarate or nitrate as the electron acceptors were able to reduce intracellularly the CTC (Smith and McFeters, 1997).

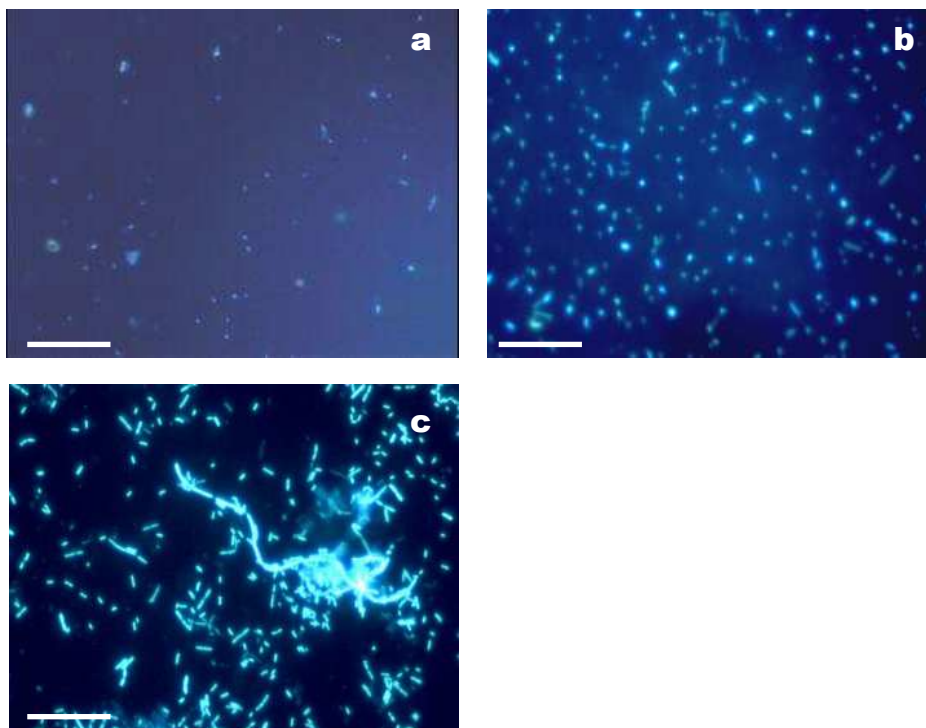


Figure 3.1 - Pictures of bacteria (blue), obtained by EF microscopy at 1000 magnification stained with DAPI, in different samples: **a** - drinking water collected directly from the tap regularly used in this study; **b** - drinking water bacteria collected from the same tap after 1 month of no-use; **c** - drinking water bacteria cultivated in a nutritive agar medium. The scale bar corresponds to 10 μm .

There is still some controversy in the research community about what exactly CTC measure. Some authors, working with aerobic bacteria, used the term respiring bacteria (Schaule *et al.*, 1993; Kalmbach *et al.*, 1997b; Schwartz *et al.*, 1998). Others working under anaerobic conditions called the bacteria viable and observed that only the dead bacteria were not able to reduce CTC (Smith and McFeters, 1997; Bhupathiraju *et al.*, 1999; Créach *et al.*, 2003). Others questioned the term active or viable bacteria after observing a rapid toxic effect of CTC on bacterial cells by inhibiting protein synthesis (Ullrich *et al.*, 1996; Servais *et al.*, 2001).

Abiotic reduction of CTC, with formazan precipitation outside the bacteria, may be possible if the medium has certain reductants, such as, ascorbic acid, cysteine, sodium sulphide, thiosulfate and iron chloride (Bhupathiraju *et al.*, 1999). This author proved also the direct transference of electrons from the coenzymes (NADH and NADPH) to tetrazolium dyes.

As described before for DAPI staining, bacteria of drinking water samples collected from the tap were generally very difficult to observe in the microscope due to the small size and the rapid decay of fluorescent signal (Figure 3.2a). On the other side, bacteria that were in the pipe immediately before the tap for 1 month (with a higher temperature than in the distribution system) had higher sizes and stronger fluorescence signals (Figure 3.2b). The same occurred with the bacteria samples were collected from the reactors fed with drinking water or with bacteria grown in nutritive media. The addition of nutrients and the temperature enhanced the metabolic activity, which corresponded to an increase in amount of CTC reduced inside the cells (and, consequently, the fluorescent signal).

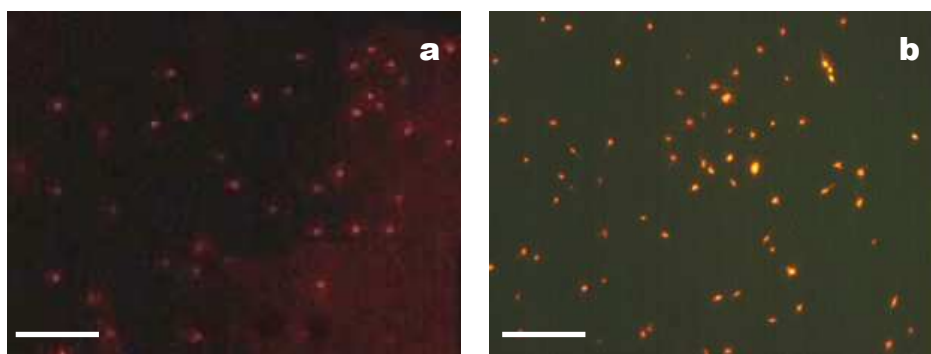


Figure 3.2 - Pictures of bacteria (red), obtained by EF microscopy at 1000 magnification stained with CTC, in different samples: a - drinking water collected from a tap regularly used in this study; b - drinking water collected from the same tap after 1 month of no-use. The scale bar corresponds to 10 μm .

As an example, Figure 3.3 presents the results of the microbiological parameters of drinking water that was flowing through the tap at 15.1 mL/day to feed continuously a biofilm monitoring reactor (**Exp. 3.2**). The total, metabolically active and the cultivable bacteria were determined and were “constant” with time within a certain range. In this assay the bacteria density was $4.4 (\pm 1.2) \times 10^5$ cells/mL in which 22 - 45 % were metabolically active and 0.05 - 1.4 % cultivable.

The slight variations observed over 54 days under the same operating conditions (continuous flow and similar daily temperatures) may be due to some experimental errors (sample collection and homogenization). However, these variations were expected as a “normal behavior” for biological systems such as this one.

Generally, the metabolically active fraction was always higher than the cultivable fraction as observed also by other authors (Schaule et al., 1993; Ultee et al., 2004),

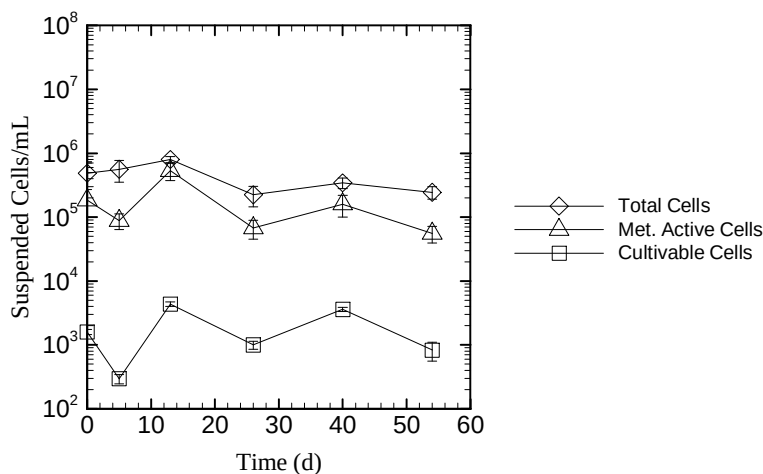


Figure 3.3 - Cell densities in terms of total, metabolically active and cultivable cells of drinking water collected from a tap providing water continuously at $Q = 15.1$ mL/day (Exp. 3.2).

probably because some bacteria are viable but not cultivable (VBNC), others are unable to form colonies per se, or even, can not grow on that specific agar medium used. VBNC state was described as a feature to promote bacteria survival during unfavorable life conditions (McDougald *et al.*, 1998; Ultee *et al.*, 2004).

In all the drinking water analyzed during the 3-years work, the bacterial density was from 0.4 to 9×10^5 cells/mL, in which 2 - 66 % were metabolically active and 0.01 - 28 % cultivable. In these ranges of the microbiological parameters, there are already included the changes attributed to the effect of stagnancy, temperature and flow, presented in the next section.

3.3.3 Effect of Stagnancy, Temperature and Flow

The effect of residence time of the stagnant water inside the pipe upstream of the tap was assessed according to the following procedure: 8 water samples of 1 L of volume were collected continuously at 1.4 L/min from a tap that was closed for 1 month (lab temperature was 28° C). The inorganic and the total organic carbon (Figure 3.4) were measured as well as the total, metabolically active and cultivable cells (Figure 3.5).

The inorganic carbon was much lower for the first sample ($p < 0.001$), and then increased to constant values around 18.9 mg C/L. The samples collected between 40 s and 3 min presented slightly higher averages than the following ones, possibly because they dragged the inorganic deposits formed in the pipe. The total organic

carbon was five times higher ($p < 0.001$) than all the following values (2.2 mg C/L) due to an increase of suspended biomass during the stagnancy period.

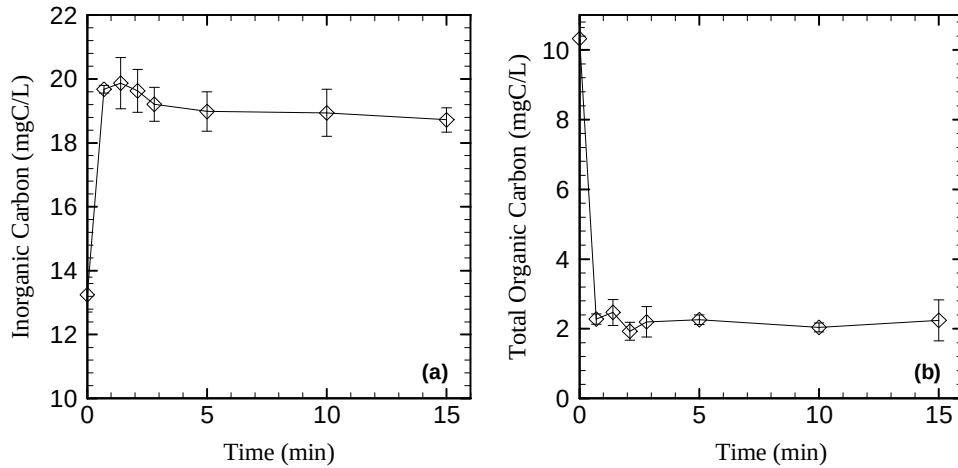


Figure 3.4 - Inorganic (a) and total organic (b) carbon of water samples collected after opening the tap at 1.4 L/min (Exp. 3.3).

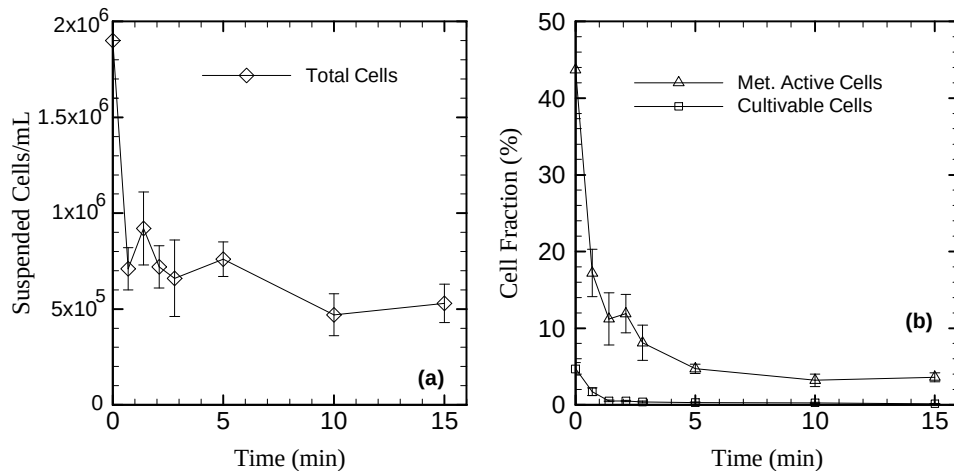


Figure 3.5 - Total cell densities (a) and fraction of metabolically active and cultivable cells (b) of water samples collected continuously after opening the tap at 1.4 L/min (Exp. 3.3).

In accordance, the total cell density of the first collected sample was much higher ($p < 0.001$) than those collected afterwards, as well as, higher values were observed for the metabolically active and the cultivable cells fractions. These two fractions may be an indicator of the cellular activity and decayed to values around 3.4 % and 0.21 %. The microscopic observation of these samples corroborated these phenomena: the cell dimensions were higher and, also, the DAPI and CTC fluorescence signals were much higher in the first sample which corresponds to

higher DNA content and dehydrogenase activity (see Figure 3.1b and Figure 3.2b). In opposition to the quick decay of the carbon content (inorganic or organic), the microbiological parameters presented a slower decay that may be attributed to biofilm removal.

The microbiological parameters of drinking water samples collected under different conditions (stagnant versus flowing water and summer versus winter) are presented in Table 3.2 (**Exp. 3.4**). Total bacteria density and the percentage of cultivable bacteria were higher in randomly sampled water in summer than in winter probably due to the differences in water temperature (around 15° and 11° C, respectively).

In the water collected after at least 5 days of continuous flow during winter, the bacteria density was approximately 3 times lower than the randomly sampled water (no permanent continuous flow, sometimes the water was stagnant). This may be due to the overall lower residence times in continuous systems and to biofilm washout. The bacterial densities in stagnant water in summer increased, probably due to higher residence time that promoted an increase in the temperature.

Table 3.2 - Microbiological parameters of Porto drinking water sampled under the indicated conditions (Exp. 3.4).

Sampling Conditions	Total Bacteria Density (Cells/mL)	Fraction of Metabolic Active Bacteria (%)	Fraction of Cultivable Bacteria (%)
Randomly sampled water during Summer (n = 7)	$4.4 (\pm 1.2) \times 10^5$	40.0 ± 12.3	0.5 ± 0.1
Sampled stagnant water between 25 and 55 days during Summer (n = 3)	$6.7 (\pm 1.1) \times 10^5$	48.4 ± 7.5	25.0 ± 3.2
Randomly sampled water during Winter (n = 7)	$2.0 (\pm 0.6) \times 10^5$	44.2 ± 5.1	0.2 ± 0.1
Sampled water after at least 5 days of continuous flow during Winter (n = 3)	$6.4 (\pm 1.5) \times 10^4$	5.7 ± 1.2	0.2 ± 0.04

3.3.4 Characterization of Microbial Populations

3.3.4.1 FISH

Fluorescent *in situ* hybridization was performed according the procedure described in section 2.5.4. In summary, drinking water bacteria were firstly fixated with formaldehyde, immobilized and dehydrated in a 10-wells plate. Then hybridization

took place after adding one FISH probe with the appropriate buffer to each well. Finally the bacteria were washed to remove the unbounded probe and stained with DAPI. After counting, the results were quantified as a percentage of the probed bacteria against the DAPI-stained bacteria. The probes and the protocol were tested initially with positive- and negative-probed bacteria. Figure 3.6 is an example of *Ralstonia basileus* and *Pseudomonas chlororaphis* that fluoresced with the BET42a labeled with Cy3 and GAM42a labeled with FLUOS, respectively.

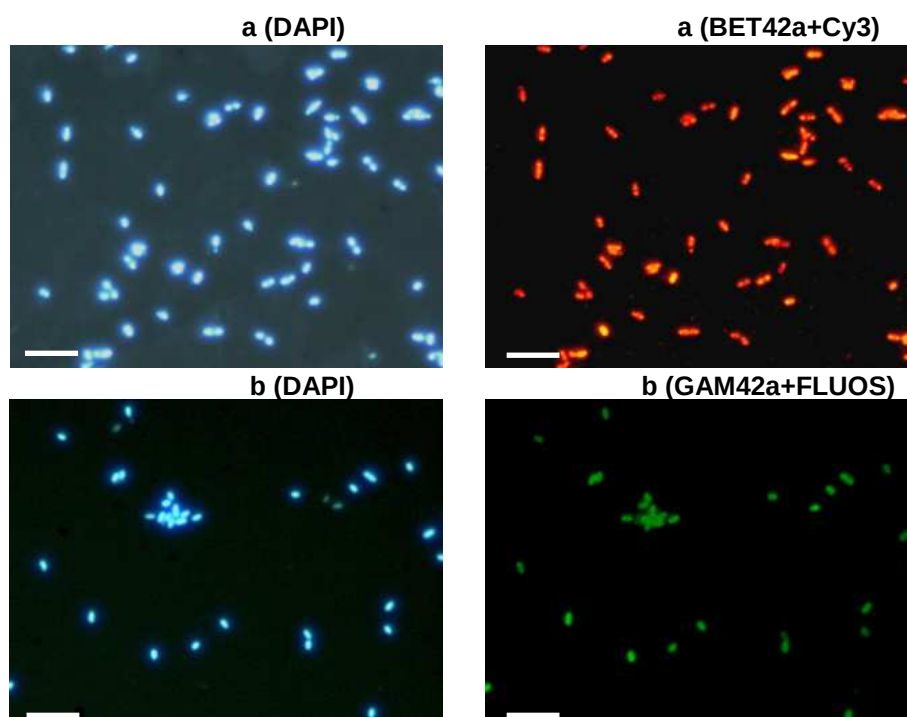


Figure 3.6 - Positive controls of FISH probes using bacteria stained with probes labeled with Cy3 (red) or FLUOS (green) and with DAPI (blue). (a) *Ralstonia basileus* with BET42a-Cy3; (b) *Pseudomonas chlororaphis* with GAM42a-FLUOS. The scale bar corresponds to 10 μm .

The application of the described protocol directly to the drinking water bacteria was not successful: the bacteria were very small, their fluorescent signal too weak and very difficult to distinguish from the signal of other particles (Figure 3.7). Another problem was the FLUOS labeled probes whose fluorescent signal was affected (intensified) by the DAPI signal after visualizing the bacteria in the blue channel. Other alternative options were tested with no improvement (triple filter block, amplification of maximum magnification by 1.4 factor and image analysis software). Therefore, the implemented modifications with success were: (i) incubating the bacteria in a nutritive medium with a DNA gyrase inhibitor

(pipemidic acid) as proposed by Kogure *et al.* (1979) and (ii) all probes were labeled only with the Cy3 dye. Other authors (Mcfeters *et al.*, 1995; Kalmbach *et al.*, 1997a; Kawai *et al.*, 1999; Kenzaka *et al.*, 2001; Baudart *et al.*, 2002; Langmark *et al.*, 2004; Baudart *et al.*, 2005; Bjergbaek and Roslev, 2005; Lee *et al.*, 2007) used this modification with success when analyzing samples from drinking water systems and achieved similar conclusions: an increase in the hybridized bacteria number and dimensions although the total cell number was constant.

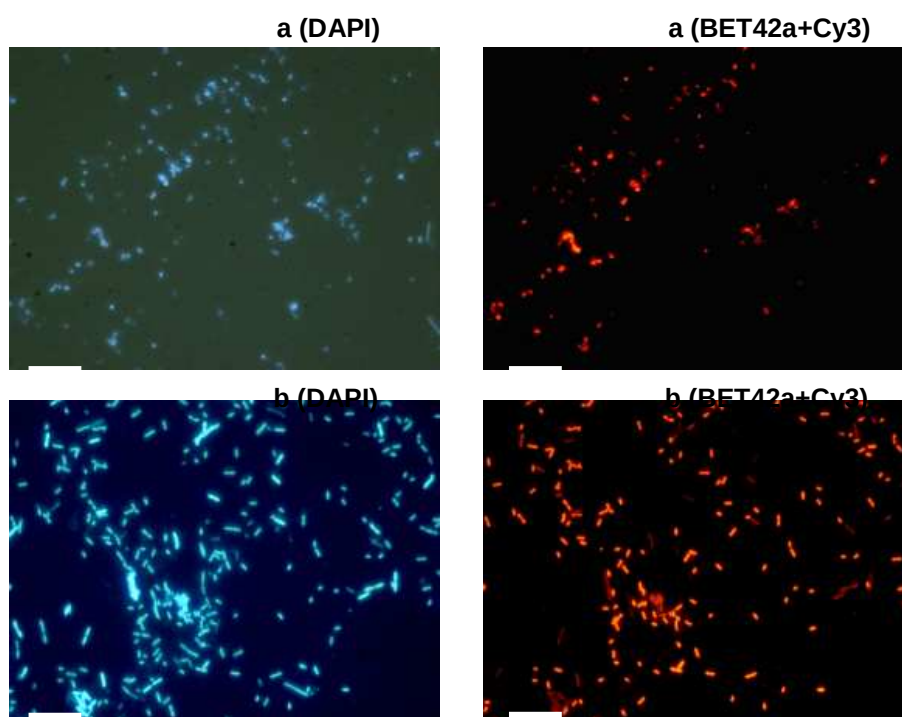


Figure 3.7 - Drinking water bacteria stained with DAPI (blue) and BET42a-Cy3 (red) probe (a) without or (b) with incubating in a nutritive medium supplemented with pipemidic acid. The scale bar corresponds to 10 μ m.

The results of FISH obtained for several drinking water samples (**Exp. 3.5**) are presented in Table 3.3. The tested probes were for the following phylogenetic groups: *Alpha-*, *Beta-* and *Gammaproteobacteria*, *Bacteroidetes*, *Actinobacteria*, ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB).

Betaproteobacteria was the most predominant phylogenetic group followed by *Gamma-* and *Alphaproteobacteria* which accounted around 72.5 % of the total cells and 90 % of the overall stained bacteria. *Bacteroidetes* and *Actinobacteria* phyla constituted 5.6 %.

Nitrite oxidizing bacteria (NOB) and ammonia oxidizing bacteria (AOB) were also investigated and less than 3.3 % was present, being AOB fraction always higher than that of NOB.

Table 3.3 - Composition of drinking water sampled in the indicated dates in terms of each targeted phylogenetic group (Exp. 3.5).

Phylogenetic groups		Fractions of phylogenetic groups (%)		
		22-02-06	08-06-06	03-03-07
<i>Bacteria</i> domain subgroups	<i>Alphaproteobacteria</i>	8.5 ± 1.2	9.2 ± 2.3	4.2 ± 1.3
	<i>Betaproteobacteria</i>	38.3 ± 6.5	43.4 ± 3.6	47.5 ± 5.1
	<i>Gammaproteobacteria</i>	19.1 ± 3.4	26.1 ± 2.5	25.3 ± 1.9
	<i>Bacteroidetes</i>	4.6 ± 2.7	2.5 ± 1.3	4.7 ± 2.4
	<i>Actinobacteria</i>	2.5 ± 1.1	2.3 ± 0.8	1.2 ± 0.7
	AOB (<i>Nitrosomonadales</i>)	3.2 ± 1.4	1.1 ± 0.6	2.7 ± 1.2
	NOB (<i>Nitrospirales</i>)	0.8 ± 0.1	1.5 ± 0.1	1.5 ± 0.1

AOB - ammonia oxidizing bacteria belonging to *Betaproteobacteria*; NOB - nitrite oxidizing bacteria.

Table 3.3 (cont.) - Composition of drinking water sampled in the indicated dates in terms of each targeted phylogenetic group (Exp. 3.5).

Phylogenetic groups		Fractions of phylogenetic groups (%)		
		04-03-07	06-03-07	13-04-07
<i>Bacteria</i> domain subgroups	<i>Alphaproteobacteria</i>	7.8 ± 1.3	3.2 ± 1.2	10.9 ± 1.8
	<i>Betaproteobacteria</i>	51.4 ± 5.7	42.3 ± 8.8	32.2 ± 2.4
	<i>Gammaproteobacteria</i>	26.1 ± 3.4	12.7 ± 1.6	26.6 ± 3.6
	<i>Bacteroidetes</i>	1.3 ± 0.2	5.9 ± 2.2	5.2 ± 1.1
	<i>Actinobacteria</i>	1.3 ± 1.0	0	2.1 ± 0.9
	AOB (<i>Nitrosomonadales</i>)	1.4 ± 0.8	1.9 ± 0.4	2.7 ± 1.7
	NOB (<i>Nitrospirales</i>)	0.2 ± 0.04	1.1 ± 0.1	1.3 ± 0.1

AOB - ammonia oxidizing bacteria belonging to *Betaproteobacteria*; NOB - nitrite oxidizing bacteria.

3.3.4.2 DGGE

Denaturing Gradient Gel Electrophoresis was performed to study the total bacterial community in drinking water samples (Exp. 3.5). Due to the low bacterial densities in tap water, it was very difficult to extract and concentrate enough total DNA from drinking water bacteria in order to observe bands in the DGGE gel. Using one PCR cycle, a very large volume (20 L) was necessary for the purpose. By

running two consecutive PCR cycles (nested PCR), only 1 L of sample was enough to obtain bands in the DGGE gel.

Figure 3.8 presents some DGGE gels obtained for drinking water samples. Gel A shows the similarities of the band patterns of DNA extracted from drinking water collected on day 17-05-2005 and amplified on different runs, indicating that PCR amplification was very reproducible.

In Gel B, B1 and B2 lanes are from the same drinking water sample collected on day 02-09-2005 and amplified through 1-cycle PCR and nested PCR, respectively. Nested PCR seemed advantageous over the PCR as band intensities increased due to higher DNA yield. Another consequence was that more bands were visualized in B11 (indicated with black arrows).

Gel C shows the band patterns of the drinking water sample collected on day 29-03-2006 that was processed in three different ways. The DNA was extracted directly and amplified (lane C1) or DNA were obtained from the colonies grown on cetrimide agar (C2) and R₂A agar (C3) after filtering 100 mL of drinking water. C4 lane was from bacteria grown in R₂A from 100 µL of sample volume. Microbial composition was much more similar among the cultivated samples in different agar media when compared to A1 indicating that the major fraction of cultivated bacteria belonged to *Pseudomonas* because cetrimide agar is a selective medium for some members of this genus. As expected, C3 pattern (from 100 mL filtrated volume) shows a more diverse bacteria community than C4 pattern (from 100 µL of sample).

The relations between band intensity *versus* DNA fragment amount and band number *versus* bacteria types are not as linear as concluded by several authors. Possible explanations were given: the co-migration of phylogenetically heterogeneous fragments in a DGGE gel (Sekiguchi *et al.*, 2001) and the PCR errors of the targeted DNA sequences when combined with the primers (Janse *et al.*, 2004). Other artifacts generated from PCR were the preferential amplification of certain sequences, the false positives due to contaminants and the generation of chimeric sequences (Dahllof and Kjelleberg, 2002; Hongoh *et al.*, 2003). Chimeras are composed of two or more phylogenetically distinct parental sequences resulting from a prematurely ended amplicon when it reanneals to a different DNA template and is copied to completion based on this second parental sequence (Hugenholz and Huber, 2003; Gonzalez *et al.*, 2005). Multiple bands from one amplified DNA sequence may be observed in the gel when the corresponding organism possesses more than one operon of that gene. For example, *Gulosibacter molinivorax* and

Achromobacter xiloxidans resulted always in four distinct bands in a DGGE gel (Barreiros *et al.*, 2008). Also, the amount of extracted DNA amount is not proportional to the bacteria concentration as it depends on cell lysis and nucleic acid extraction efficiency.

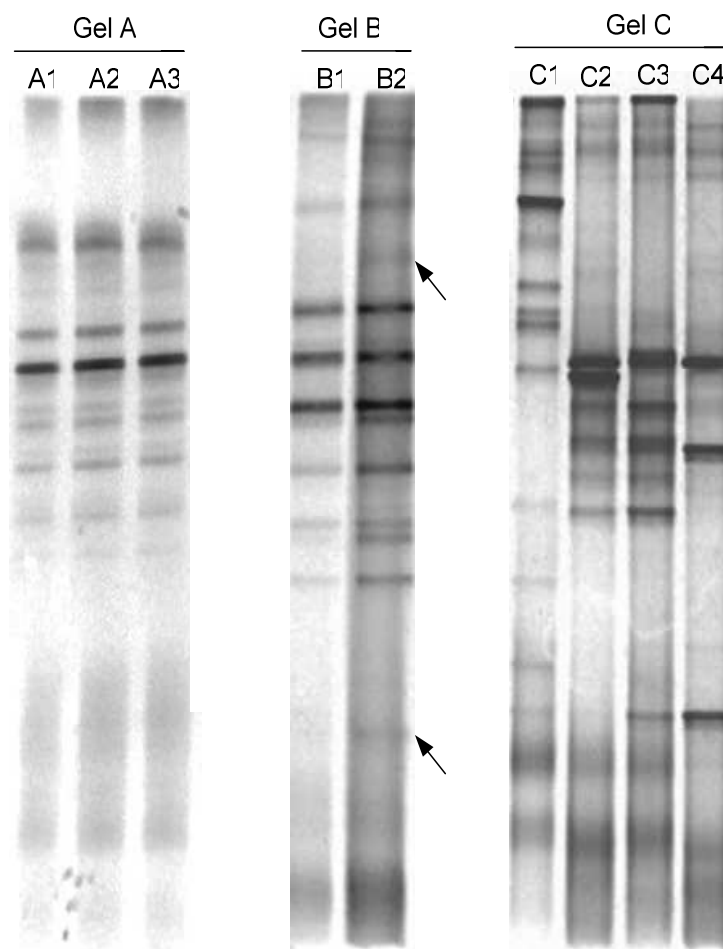


Figure 3.8 - DGGE patterns from 16S rDNA 180 bp fragments of (Exp. 3.5): **Gel A** - the same drinking water sample (collected on day 17-05-2005) and amplified in different runs; **Gel B** - the same drinking water sample (collected on day 02-09-2005) and then amplified by PCR (B1) and nested PCR (B2). The arrows indicate the bands observed in B2 that are not visualized in B1; **Gel C** - the same drinking water sample (collected on day 29-03-2006) in which the DNA was immediately extracted and amplified (C1) or after cultivation in cetrimide (C2) or R₂A agar (C3 and C4).

3.3.4.3 Phenotypic Characterization

The phenotypic characterization with API 20NE kit (non-enteric and non-fermenting gram-negative rods) was performed for the cultivated fraction of

bacteria from three independent water samples (**Exp. 3.6**) collected on days 03-07-2003, 20-01-2004 and 16-06-04. However, it should be emphasized that this identification system has several limitations. For example, different results may be obtained upon repeated testing and the database is limited to 61 different taxa. Bosshard *et al.* (2006) concluded from his comparative studies with API 20 NE and 16S rRNA gene sequencing that the former test was reliable when the species assignment was reported with excellent or very good identification. Thus, only the bacteria identified with an excellent or a very good classification are going to be presented.

The drinking water samples were cultivated in R₂A and all the different morphotypes were purified through sub-culturing on the same medium. For the three drinking water samples, all isolates were rod-shaped and the major fraction (90.3 %) was Gram-negative (Table 3.4). This fact is in accordance with the results from FISH that showed that *Proteobacteria* was the dominant group while the Gram-positive bacteria (*Actinobacteria*) were present as a minor fraction. Different results were observed by Pepper *et al.* (2004) that obtained only 0.2 % of Gram-negative bacteria from tap water due to chlorination. This effect of the disinfectant was also observed by Norton and LeChevalier (2000) although the Gram-negative bacteria were always the major fraction.

The Gram-negative and oxidase-positive (non-fermenters) isolates were identified with the API 20 NE kit and the results are presented in Table 3.4. The presence of members of *Pseudomonas*, *Xanthomonas* and *Stenotrophomonas* were expected since these genera belong to *Gammaproteobacteria*, a phylogenetic group that constituted around 12.7 - 26.6 % of the total cells (FISH analysis). Although *Bacteroidetes* and *Alphaproteobacteria* were present in low percentages, some isolates were affiliated to their respective genera *Flavobacterium* and *Sphingomonas*. In opposition, *Betaproteobacteria* was the most common group as observed with FISH, however, none of the isolates could be affiliated to members of this subclass, showing that the cultivable microbial population does not reflect the total microbial diversity of the drinking water. The results obtained are in accordance to those reported by other authors, describing the presence of *Flavobacterium*, *Alcaligenes*, *Methylobacterium*, *Pseudomonas* and *Acinetobacter* species in cultivated drinking water samples (Hirata *et al.*, 1993; Penna *et al.*, 2002).

Some non-fermentative Gram negative rods are considered opportunistic pathogens responsible for nosocomial infections. Infections due to *Pseudomonas aeruginosa*,

Table 3.4 - Cultivated bacteria identified in three independent samples of drinking water (Exp. 3.6).

	Drinking water samples		
	03-07-03	20-01-04	16-06-04
Dilution factor	1	0	1
Gram-negative isolates	90.9 % (10/11)	90.0 % (9/10)	90.9 % (9/10)
<i>Pseudomonas vesicularis</i>	---	10.0 % (1/10)	---
<i>Pseudomonas cepacia</i>	9.1 % (1/11)	---	20.0 % (2/10)
<i>Xanthomonas</i> sp.	---	10.0 % (1/10)	---
<i>Flavobacterium</i> sp.	9.1 % (1/11)	10.0 % (1/10)	---
<i>Sphingomonas paucimobilis</i>	18.2 % (2/11)	10.0 % (1/10)	10.0 % (1/10)
<i>Pseudomonas</i> sp.	9.1 % (1/11)	20.0 % (2/10)	10.0 % (1/10)
<i>Stenotrophomonas maltophilia</i>	18.2 % (2/11)	---	10.0 % (1/10)
Non identified Gram-negative isolates	27.3 % (3/11)	30.0 % (3/10)	40.0 % (4/10)
Gram-positive isolates	9.1 % (1/11)	9.0 % (1/10)	9.0 % (1/10)

Burkholderia cepacia, *Stenotrophomonas maltophilia* or *Acinetobacter* species, usually hospital-acquired ones, may be lethal in debilitated patients, for example those in whom invasive devices were used (Berthelot *et al.*, 2005; Paterson, 2006; Enoch *et al.*, 2007).

3.3.4.4 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic pathogen, widespread in the environment that can grow and accumulate in drinking water distribution systems where was detected by several authors (Penna *et al.*, 2002; Kilb *et al.*, 2003; Emtiazi *et al.*, 2004).

Reports (Empel *et al.*, 2007; Pitout *et al.*, 2007) were published about health risk events occurring specially in hospitals. This species is resistant to almost all commercially available antibiotics and can produce serious nosocomial infections if it gains access to the body through wounds or intravenous lines (Berthelot *et al.*, 2005; Paterson, 2006). Forty-three outbreaks of waterborne nosocomial infections have been reported until 2001 (Anaissie *et al.*, 2002), and an estimated 1400 deaths occur each year in the United States as a result of waterborne nosocomial pneumonias caused by *Pseudomonas aeruginosa* alone.

Two independent experiments in the Flow Cell reactor fed with tap water, recirculating with a hydraulic residence time of 12 h, resulted in water coloration and odor changing. When the water reactor and biofilm samples were cultivated, colonies revealing the same characteristics (acrid odor and dark-green color) of *Pseudomonas aeruginosa* were found. For this reason, 16 samples of 1 L of tap water with were collected (**Exp. 3.7**) and cultivated in selective medium. *Pseudomonas aeruginosa* was not presumptively detected in any of these samples. However, it is possible that this species was present in tap water but appeared so occasionally that was not detected in the collected samples.

3.4 Conclusions

- a) Porto drinking water was practically neutral with low contents of dissolved solids, of organic carbon, of phosphorous, of nitrogen and of chlorine. These values are within the admissible range legislated in the Portuguese Law nº 176/98.
- b) Tap water contained around $2 - 5 \times 10^5$ cells/mL, 22 - 45 % of them being metabolically active and 0.05 - 1.5 % cultivable. These parameters were affected by the stagnancy, the temperature and the flow. High microbial densities were found in the first water collected after a stagnant period. During summer, total cell densities were twice as higher as in winter. In winter time, cell densities decreased when the tap was continuously opened.
- c) FISH technique was implemented successfully for the drinking water samples although incubation was needed in a nutritive medium supplemented with an antibiotic. Drinking water samples were mostly composed *Proteobacteria*, being *Beta*-subclass predominant. In minor fractions, members of *Bacteroidetes* and *Actinobacteria* phyla were also present in the drinking water samples as well as AOB and NOB.
- d) DGGE was implemented successfully and with reproducibility for the drinking water samples that presented complex and diversified microbial patterns. Nested PCR was advantageous over PCR as higher DNA copies were generated accurately.
- e) After cultivation, almost all bacterial isolates of drinking water were Gram-negative rods (90.3 %). The majority of these isolates were affiliated to genera *Pseudomonas*, *Xanthomonas*, *Stenotrophomonas*, *Sphingomonas* and *Flavobacterium*.

f) *Pseudomonas aeruginosa* was not detected in any of the samples of drinking water.

4. Biofilm Growth under Steady State Hydraulic Conditions

4.1 Introduction

In drinking water systems, the hydraulic regimes may range from turbulent to laminar flow, and stagnant periods may occur when water demand is low. This chapter intends to elucidate the effect of these regimes on biofilm accumulation. Experiments were performed using one of the most common polymeric material (PVC) as biofilm adhesion surfaces in different monitoring reactors (Propella, Flow Cell and Batch reactors) under continuous flow (from laminar to turbulent) and in stagnant systems. Cell accumulation inside each reactor was analyzed after a few hours of operation until 40-50 days. The specific growth rates of suspended and attached cells were also quantified. The effects of flow velocity and residence time on the kinetic parameters were evaluated. The work presented in this chapter was partially published in the following scientific paper: **Manuel, C. M., Nunes, O. C. and Melo, L.F. (2007) Dynamics of drinking water biofilm in flow/non-flow conditions, Water Research, 41, 551-562.**

4.2 Experimental Methodology

Reactors. Biofilm monitoring systems used to test the biofilm accumulation were a Batch reactor, a Propella reactor and a Flow Cell reactor, their main characteristics being described in section 2.1.

Biofilms were formed on coupons made of PVC placed within the reactors in contact with drinking water under different operating conditions, including continuous flow and stagnant systems.

The Propella and Flow Cell reactors were connected to the drinking water distribution system in the town of Porto and the same water was used to fill up the Batch reactor.

Operating conditions. In the first set of assays (**Exp. 4.1**), Propella, Flow Cell and Batch reactors were operated for 56 days. The continuous flow reactors were fed with drinking water at appropriate inlet flow rates (Q_{ext}) in order to obtain equal hydraulic residence times (HRT) and internal velocities (u_{int}) and similar wall shear stresses (τ_w) in both systems (Table 4.1). The Batch reactor was filled with drinking water and kept in the dark for 56 days. Biofilms grown on PVC and reactor water samples were collected at predefined days. Several physical, chemical and microbiological parameters were measured, such as total, metabolically active and R_2A -cultivable cell numbers, total and dissolved organic carbon, pH, total dissolved solids and temperature according the protocols described in Chapter 2. The effect of the continuous flow (Propella and Flow Cell reactors) *versus* stagnant water (Batch reactor) regimes on biofilm growth was assessed.

A second experiment (**Exp. 4.2**) was performed only in the Flow Cell reactor with a higher internal flow rate (and consequently, higher shears stresses). The operating conditions are also presented in Table 4.1. In the first days of this experiment, biofilm and water samples were collected every 12 hours. From day 5 up to day 28, samples were collected at a rate of one per week. The same parameters were determined as the ones indicated for **Exp. 4.1**. From this experiment, the initial stages of biofilm growth were observed, as well as the effect of shear stress under turbulent flow regime. Cell numbers obtained were used to determine biofilm growth rate.

In **Exp. 4.3**, the Flow Cell reactor was operated with 2 units in parallel with recirculating water at 1 and 6 L/min each and for 12 h of residence time inside the reactor (Table 4.1). Twenty sampling points of biofilm were collected from the first

day to the 39th day in order to study the effect of shear stress on kinetic parameters. The same parameters were determined as those indicated for **Exp. 4.1** except the microbiological parameters of the reactor water (total, metabolically active and cultivable cells per milliliter).

Another experiment was performed to assess the effect of recirculating water in the reactor (**Exp. 4.4**). In this case a Flow Cell unit without recirculation was connected directly to the tap at 1 L/min and biofilm accumulation was followed for 46 days. The same parameters were measured for the biofilm and the reactor water samples as those indicated for **Exp. 4.1**.

Table 4.1 - Operating conditions in Exp. 4.1, 4.2, 4.3 and 4.4 in the biofilm monitoring reactors (Propella, Flow Cell and Batch).

Reactor parameters	Exp. 4.1			Exp. 4.2	Exp. 4.3		Exp. 4.4
	Propella	Flow Cell	Batch reactor	Flow Cell	Flow Cell	Flow Cell	Flow Cell, no recirculation
Coupon material	PVC	PVC	PVC	PVC	PVC	PVC	PVC
u_{int} (m/s)	0.20	0.21	0	0.34	0.04	0.24	0.04
Q_{ext} (mL/day) or *(L/min)	7.8	15.1	0	15.1	15.1	15.1	1.0*
Reynolds No.	3970	5000	0	8293	1001	5797	1001
τ_w (Pa)	0.80	0.80	0	1.91	0.0137	1.1	0.0137
HRT (h) or *(s)	11.9	12.0	1340	12.0	12.0	12.0	20.5*

Temperature was not controlled inside the reactors since the purpose was to observe the biofilm growth under the conditions prevailing in the actual drinking water system of Porto, whose characteristics were described in the previous Chapter.

4.3 Results and Discussion

4.3.1 Reactor Water

In **Exp. 4.1**, water samples from the three reactors were regularly collected and analyzed at time zero and at different times between operation days 5 and 56. The physicochemical parameters (Table 4.2) presented almost constant values over time. The slightly lower temperatures registered in the Propella are due to the fact that

this reactor was installed in a colder room. In **Exp. 4.2** and **4.3**, reactor water samples were also analyzed every time biofilm samples were collected and the measured physicochemical parameters of the reactor water were generally similar to those of Flow Cell in **Exp. 4.1** (Table 4.2). These values were similar to the ones found in tap water, as presented in Table 3.1 except the temperature and the TOC. These two parameters were slightly higher, which is attributed to higher residence time and higher room temperatures.

In **Exp. 4.4**, the physicochemical parameters of the reactor water were similar to the tap water values (the reactor water was not recirculating; HRT = 20.5 s).

Table 4.2 - Physicochemical parameters of reactor water samples collected in assays Exp. 4.1, 4.2, 4.3 and 4.4 in the biofilm monitoring reactors (Propella, Flow Cell and Batch).

Parameters	Exp. 4.1			Exp. 4.2	Exp. 4.3	Exp. 4.4
	Propella	Flow Cell	Batch	Flow Cell	Flow Cell	Flow Cell, no recirculation
T (°C)	18.5 ± 2.1	21.0 ± 1.4	20.2 ± 1.0	22.3 ± 1.3	20.5 ± 1.1	13.0 ± 0.7
pH	7.4 ± 0.1	7.4 ± 0.1	7.4 ± 0.1	7.5 ± 0.2	7.5 ± 0.2	7.7 ± 0.3
O ₂ (mg O ₂ /L)	7.6 ± 0.9	6.2 ± 1.1	7.2 ± 1.1	n.d.	n.d.	n.d.
IC (mg C/L)	16.3 ± 0.1	16.2 ± 0.1	15.5 ± 0.1	16.7 ± 0.6	n.d.	14.7 ± 0.8
TOC (mg C/L)	2.1 ± 1.3	3.9 ± 1.1	3.2 ± 0.7	3.6 ± 1.8	n.d.	1.9 ± 1.5
DOC(mg C/L)	2.1 ± 1.0	3.77 ± 0.9	3.3 ± 0.7	3.4 ± 0.5	n.d.	n.d.
TDS (mg/L)	174 ± 29	165 ± 8	172 ± 7	158 ± 13	164 ± 11	152 ± 10

IC - inorganic carbon; TOC - total organic carbon; DOC - dissolved organic carbon; TDS - total dissolved solids; n.d. - not determined. All values are given as mean ± standard deviation of the data registered over the operation days.

The densities of cells in the reactor water of the three reactors and in the feeding tap water for **Exp. 4.1** are plotted in Figure 4.1. Tap water values are presented just to make easier the analysis and discussion.

In all reactors, a quasi-steady state was achieved after 5 operation days, since suspended cell densities were thereon constant with time, although with some fluctuations. The cell densities decreased in the order of total, metabolically active and R₂A-cultivable cells for each water system tested. Around 35 - 50 % of the total cells were metabolically active and fewer (5 - 20 %) were able to form colony units in R₂A medium.

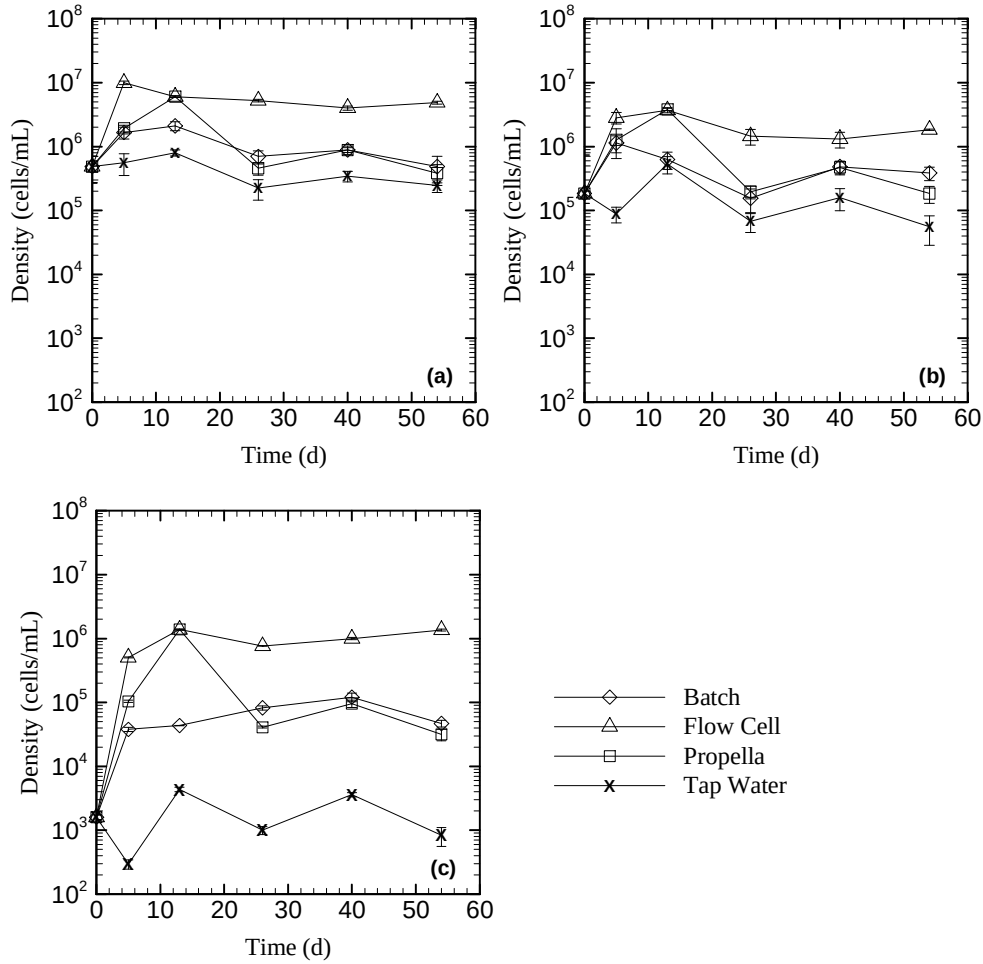


Figure 4.1 - Cell densities in reactor water samples: (a) - total cells; (b) - metabolically active cells and (c) - R_2A -cultivable cells for the indicated biofilm monitoring reactors in **Exp. 4.1** and in tap water.

In **Exp. 4.1**, the total cell numbers per milliliter in the reactor water were ten fold higher in the Flow Cell than in the Propella reactor.

Total suspended cell density in the Batch reactor was lower than in the Flow Cell, since there is no additional feed of biomass or nutrients after loading the reactor. As expected, inlet tap water presented the lowest values of suspended cells, since residence times and temperatures are higher inside the reactors.

In the Flow Cell assay with a higher internal velocity (**Exp. 4.2**), a high initial increase of suspended cells was observed and, after 2-3 days of operation, the steady state was reached with $1.8 \times 10^7 \pm 3.1 \times 10^6$ total suspended cells/mL (see Figure 4.2). This value is around three times higher than that observed in **Exp. 4.1**

inside the Flow Cell reactor, which was $6.1 \times 10^6 \pm 3.1 \times 10^5$ total suspended cells/mL. The increase in the reactor water cell densities with internal velocity may indicate a higher detachment rate and a possible higher planktonic growth due to an increase of the average temperature.

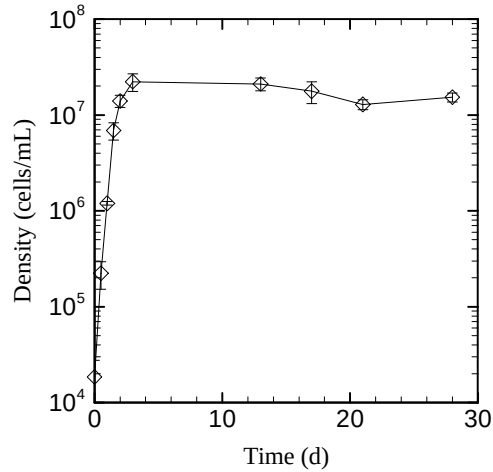


Figure 4.2 - Total cell densities in reactor water samples collected in **Exp. 4.2**.

In **Exp. 4.4** (Figure 4.3), the cell densities in the reactor water were similar to those found in tap water and slightly different from those observed in experiments with higher residence time (**Exp. 4.1 and 4.2**) (high initial increase followed by a plateau). The fractions of metabolic active and cultivable cells were also similar to those in tap water in the range of 4 - 40 % and 0.02 - 2 %, respectively.

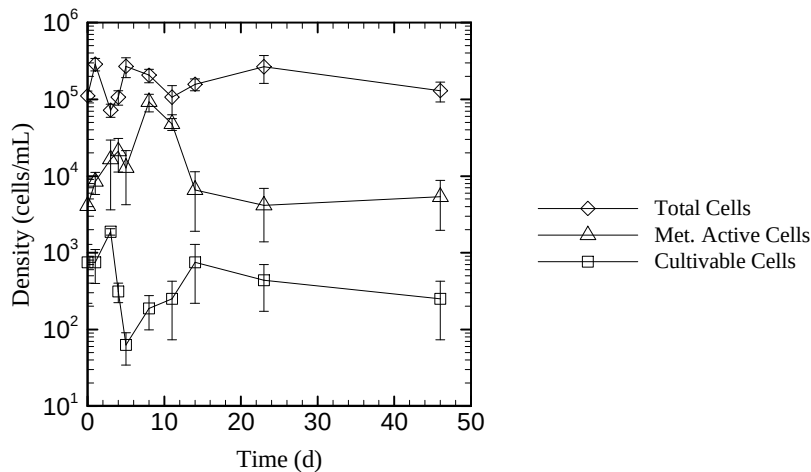


Figure 4.3 - Densities of total, metabolic active and cultivable cells in reactor water samples collected during **Exp. 4.4**.

4.3.2 Biofilm Cells - Total, Metabolically Active and Cultivable

Biofilm samples were collected during the quasi-steady state phase for microbiological analysis and the results are presented in Figure 4.4 for assays with the Propella, Flow Cell and Batch reactors containing the PVC coupons (**Exp. 4.1**).

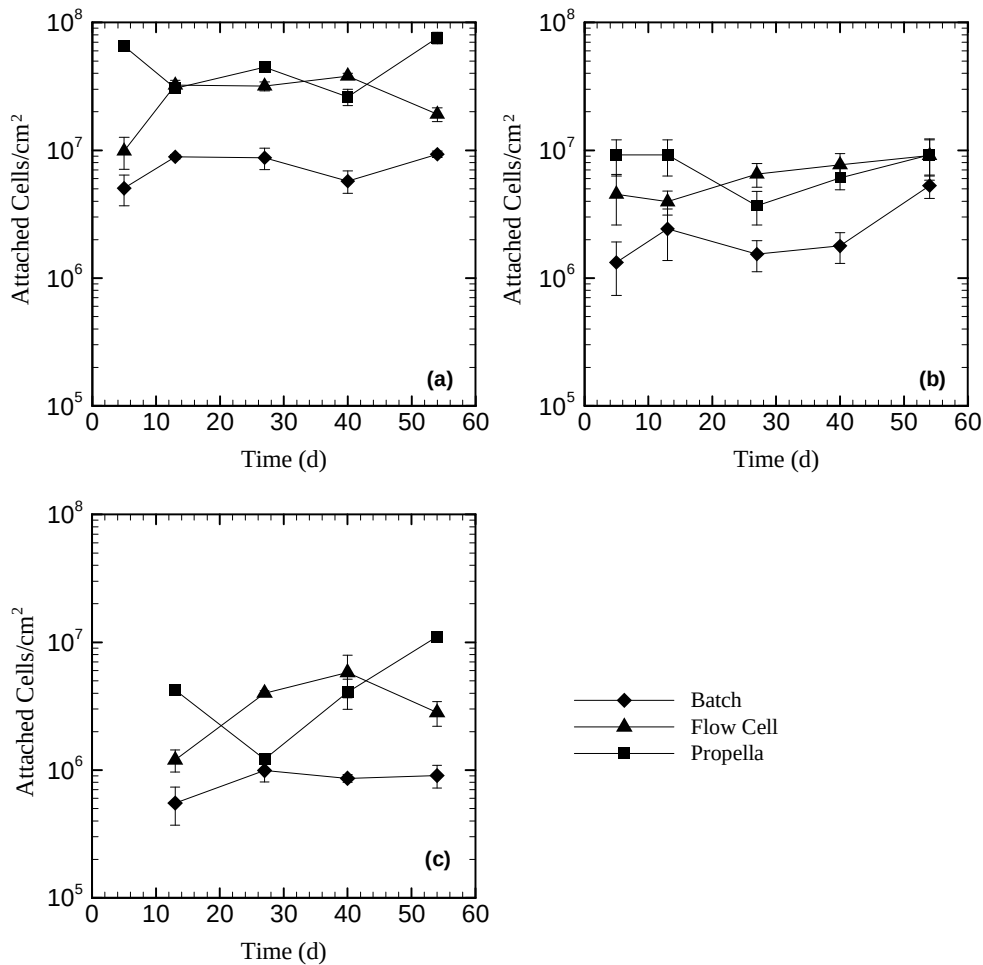


Figure 4.4 - Attached cells per unit surface area of the biofilm grown on PVC in **Exp. 4.1** defined as: (a) - total cells, (b) - metabolically active cells and (c) - R₂A-cultivable cells.

The values of total, metabolically active and R₂A cultivable biofilm cells obtained in the continuous reactors (Propella and Flow Cell) were similar [$3.7 (\pm 0.2) \times 10^7$ cells/cm²] and higher than in the Batch system [$7.5 (\pm 0.5) \times 10^6$ cells/cm²]. This could be due to the fact that in the continuous systems the dilution rate was much higher than the microbial growth rates, which is known to stimulate adhesion (Heijnen *et al.*, 1992).

As in the case of suspended cells, the biofilm total cells per square centimeter were always higher than the metabolically active cells and these were higher than the cultivable cells grown on PVC at each sampling time. Similar results were found by several authors (Schaule *et al.*, 1993; Kalmbach *et al.*, 1997b; Schwartz *et al.*, 1998; Boe-Hansen *et al.*, 2002a; Wingender and Flemming, 2004). Around 17 - 35 % of the total cells were metabolically active and 8 - 15 % was cultivable in R₂A medium. These percentages of metabolically active cells are within the reported ranges (Schaule *et al.*, 1993; Kalmbach *et al.*, 1997b; Schwartz *et al.*, 1998). The data on cultivable cells agree with those published by Schwartz *et al.* (1998), who studied biofilms of 9 - 36 days, similar to those reported in the present paper (5 - 56 days). All the other authors based their work on much older biofilms (6 months - 99 years), which may explain the loss of cultivability of cells (less than 4 % of cultivable cells).

In **Exp. 4.2** (Figure 4.5) and in **Exp. 4.4** (Figure 4.6), biofilm samples were collected from the first day presenting increasing cell numbers until a plateau was reached at the 2nd or 3rd day. For **Exp. 4.2**, with the higher flow velocity, the steady state values were around 1×10^7 bacteria/cm², in which constant fractions of cultivable cells were obtained in the range of 4.6 - 17.7 %. In **Exp. 4.3**, only the total cells per square centimeter were obtained with time and presented the same trend line: initial increase during 3 days followed by a plateau. This data is included in the next section for the analysis of effect of velocity on biofilm accumulation.

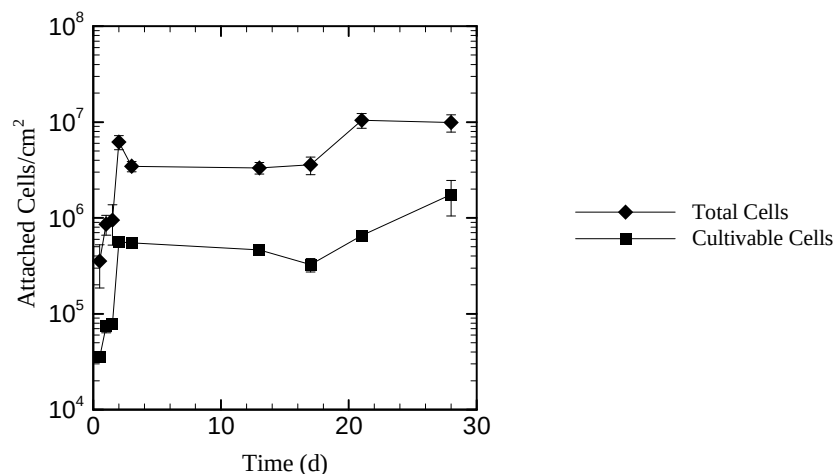


Figure 4.5 - Attached cells per unit surface area of the biofilm grown on PVC at $u = 0.34$ m/s and HRT = 12 h in **Exp. 4.2** defined as total and R₂A-cultivable cells.

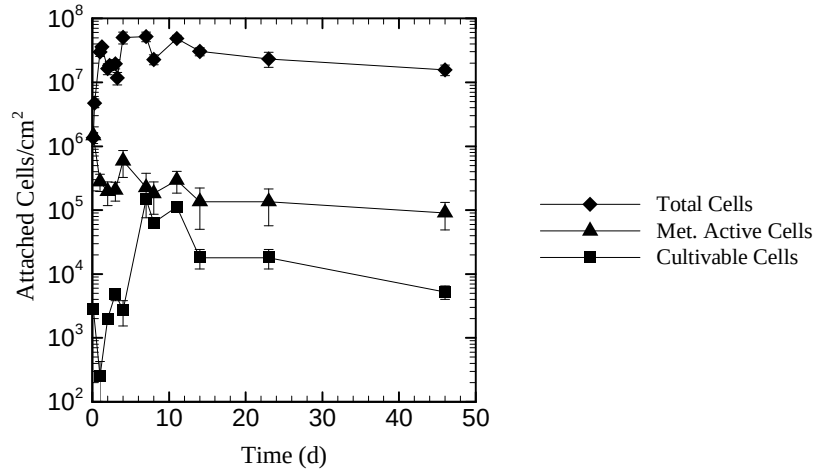


Figure 4.6 - Attached cells per unit surface area of the biofilm grown on PVC at $u = 0.04$ m/s and $HRT = 20.5$ s in **Exp. 4.4** defined as total, metabolically active and R_2A -cultivable cells.

In **Exp. 4.4**, the water velocity was much lower (0.04 m/s) as was probably the detachment rate, leading to higher steady state bacteria numbers in the biofilm (4×10^7 cells/cm²). Therefore, the fraction of metabolically active bacteria was around 100 % in the first sampling point (0.125 day) and then decreased to constant values around 0.5 - 1.0 %. Cultivable bacteria were always lower than the metabolically active ones and constant over time (lower than 0.5 %). When compared to the values obtained in **Exp 4.2**, these lower fractions of cultivable bacteria may be attributed to the lower residence time (no recirculation) and reactor temperature.

4.3.3 Effect of Flow Velocity and Residence Time

Figure 4.7 compares the total cell numbers per unit surface area for biofilms grown on PVC under different flow velocities (**Exp. 4.2** and **4.3**). The increase of velocity (from 0.04 to 0.034 m/s) resulted in lower cell numbers per unit surface area which may be explained by higher detachment rates. This effect has been frequently observed in biofilms formed under turbulent flow conditions (Pinheiro *et al.*, 1988; Bott, 1995; Soini *et al.*, 2002; Cloete *et al.*, 2003).

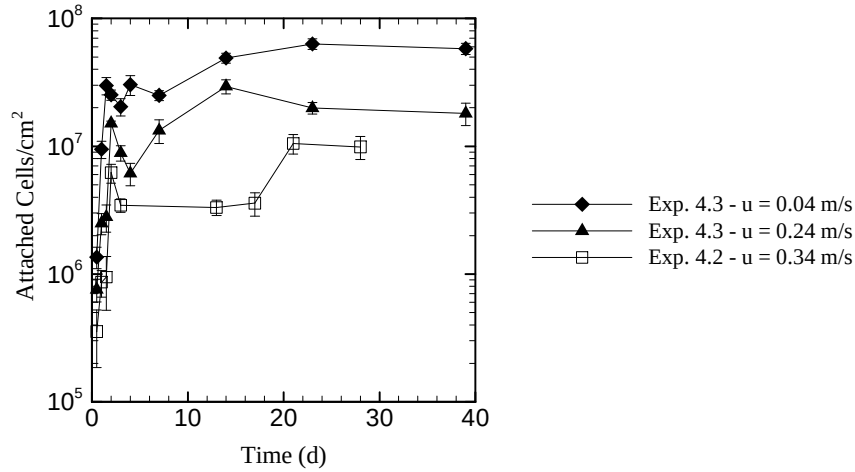


Figure 4.7- Total attached cells per unit surface area of biofilm grown on PVC in the Flow Cell with different flow velocities (0.04, 0.24 and 0.34 m/s). The residence time for **Exp. 4.2** and **4.3** was 12.0 h.

Biofilm accumulation on PVC under laminar flow, with low and high residence time of reactor water (20.5 s and 12.0 h) is presented in Figure 4.8. The obtained values were similar until 15th day despite some oscillations. After that period, the steady state biofilm presented higher values for the assay with HRT = 12.0 h, which may be attributed to higher temperature and higher HRT (and consequently higher suspended cells per mL).

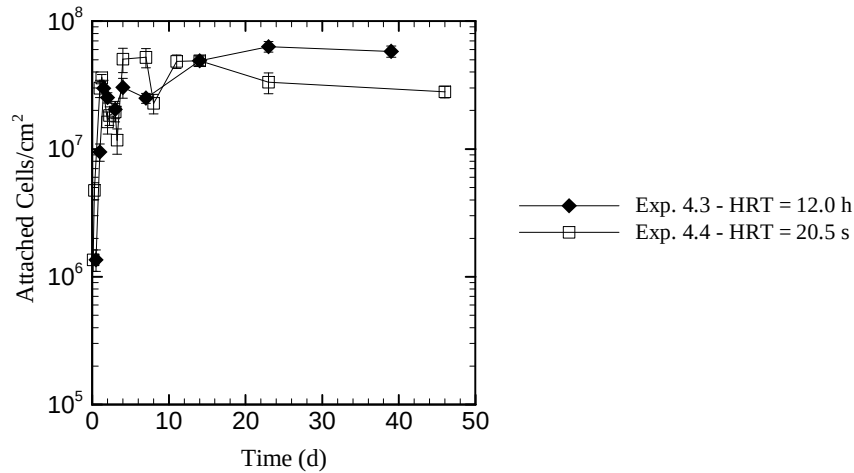


Figure 4.8 - Total attached cells per unit surface area of biofilm grown on PVC in the Flow Cell with different residence times (20.5 s and 12.0 h) and for the same velocity (0.04 m/s) - **Exp 4.3** and **4.4**.

4.3.4 Suspended Cells versus Biofilm Cells

In Figure 4.9, the total attached cells per unit surface area are plotted against the total suspended cell density for biofilms grown on PVC at quasi-steady state in **Exp. 4.1**. Apparently, no correlation was detected between suspended and biofilm total cells.

It is interesting to note that the average ratio between attached and suspended cells in the quasi-steady state period depended on the reactor type, being much higher in the Propella system (about 50) than in the Flow Cell (about 10), cf. Figure 4.10. Two factors may explain this behavior: firstly, the average temperature is higher in the Flow Cell; secondly, although equal water velocities were used in the Propella and Flow Cell reactors, the Reynolds number was 25% higher in the Flow Cell system, which should indicate a higher degree of turbulence intensity in this reactor (this is due to differences in the hydraulic diameter and cross section shape of the two reactors which imply different hydrodynamic patterns). For the Batch reactor, the average ratio between the attached and the suspended cell numbers was similar to the Flow Cell. Although the reactor water was stagnant (null velocity) in the Batch reactor and consequently no shear stress-induced detachment, the temperature was in the same range.

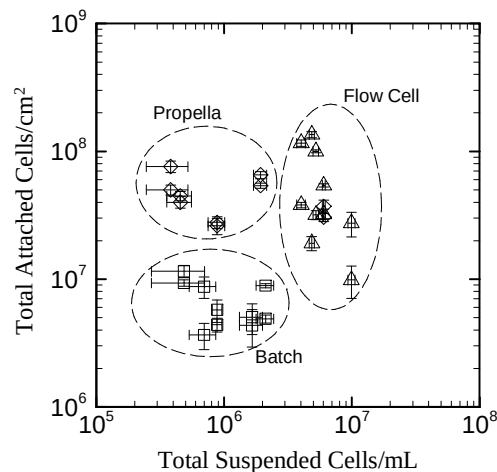


Figure 4.9 - Total attached cells per unit surface area versus total suspended cell density in **Exp. 4.1** in Propella, Flow Cell and Batch reactors.

Figure 4.10 presented the results in steady state obtained in the Flow Cell under different flow velocities and residence time. A lower attached cells/suspended cells ratio was found when a higher velocity was used in the same reactor (Flow Cell,

Exp. 4.1 and 4.2) and the same HRT (Figure 4.10). This may be due to an increase in the flow velocity (hence, shear stress) and Reynolds number (and, therefore, degree of turbulence) from Exp. 4.1 to Exp. 4.2 in the Flow Cell.

The simultaneously effect of lowering the velocity to 0.04 m/s and the residence time to 20.5 s (**Exp. 4.4**) increased the attached cells/suspended cells ratio to much higher values in the Flow Cell reactor (from 10 to 500). This may be attributed to the fact that lower HRT leads to lower reactor temperature and consequently lower suspended bacteria growth. On the other side, the removal of attached bacteria decreased for lower velocities thus increasing biofilm numbers.

The data in Figure 4.10 also suggest that in steady-state the attached cells number is not controlled by the suspended cells number, confirming previous works (Bott and Miller, 1983; Melo and Vieira, 1999).

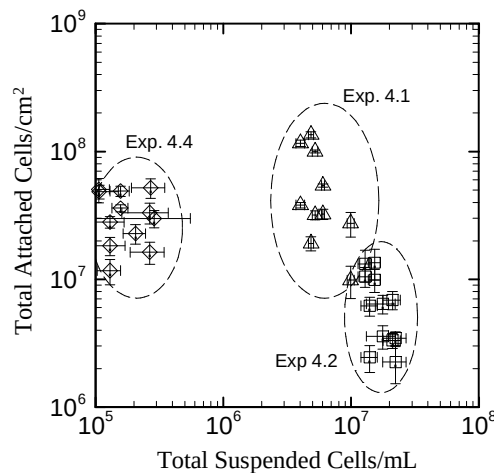


Figure 4.10 - Total attached cells per unit surface area of biofilm versus total suspended cell density in Flow Cell for: **Exp. 4.1** ($u = 0.21$ m/s, HRT = 12.0 h), **Exp. 4.2** ($u = 0.34$ m/s, HRT = 12.0 h) and **Exp. 4.4** ($u = 0.04$ m/s, HRT = 20.5 s).

Although suspended cells may still attach to the surface and participate to the biofilm formation, their contribution seems to have a minor effect when compared to the internal growth of the attached cells. Nutrients, chemical parameters (pH, biocides, etc.) and hydrodynamics seem to be the most important factors that condition biofilm growth rates (Pedersen, 1990; Bott, 1995; Kerr *et al.*, 1999; Batté *et al.*, 2003c), except when there is significant release of nutrients from the surface material to the water (Lehtola *et al.*, 2004; Lehtola *et al.*, 2005).

4.3.5 Growth Kinetics

In order to assess the kinetics of biofilm development and compare with the suspended cell growth rates, tests were carried out in the Flow Cell (**Exp. 4.2 to 4.4**) where a higher number of biofilm and water samples were collected during the initial phase (transient period) of biofilm formation. The results are presented in Figure 4.11 for biofilm grown on PVC (**Exp. 4.2**) and in Figure 4.12 for biofilms grown under different flow velocities (**Exp. 4.3**) and different residence time (**Exp. 4.4**).

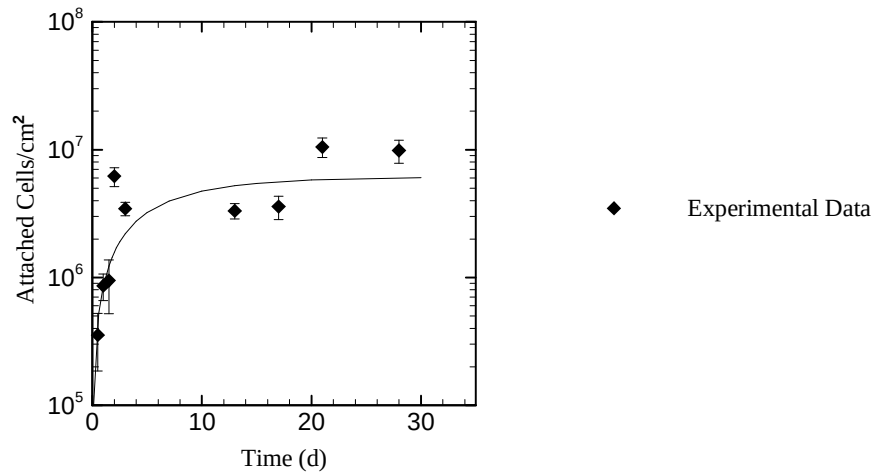


Figure 4.11 - Attached cells per unit surface area of biofilms grown under $u = 0.34$ m/s and HRT = 12.0 h on PVC in **Exp. 4.2**. Eq.(2.21) is represented by the continuous line.

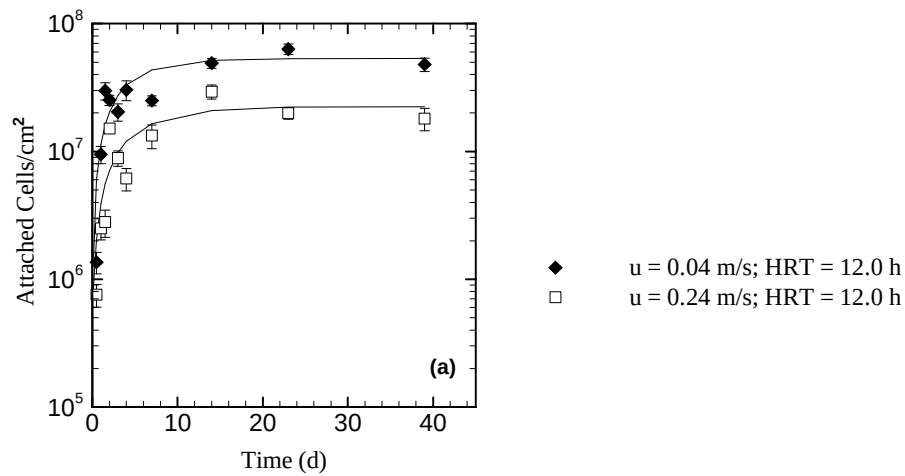


Figure 4.12 - Biofilm cells per unit surface area of biofilms grown on PVC under the indicated flow velocity and residence time in: (a) - **Exp. 4.3** and (b) - **Exp. 4.4**. Eq. (2.21) is represented by the continuous line.

The data from **Exp. 4.2, 4.3** and **4.4** was used to calculate specific growth rates in the biofilm, as presented in Table 4.3 and Table 4.4. The maximum total cell number per unit surface area, $(X_b)_{\max}$ was obtained from data at steady state (Figure 4.12) and the biofilm production rate, Φ_p , was determined from the data obtained in the initial period of biofilm formation by fitting Eq. (2.21), which allowed μ^*_{biofilm} to be calculated with Eq. (2.24). Finally, μ^*_{bulk} was calculated with Eq. (2.26) and the overall specific growth rate, μ^*_{overall} , was calculated using Eq. (2.28).

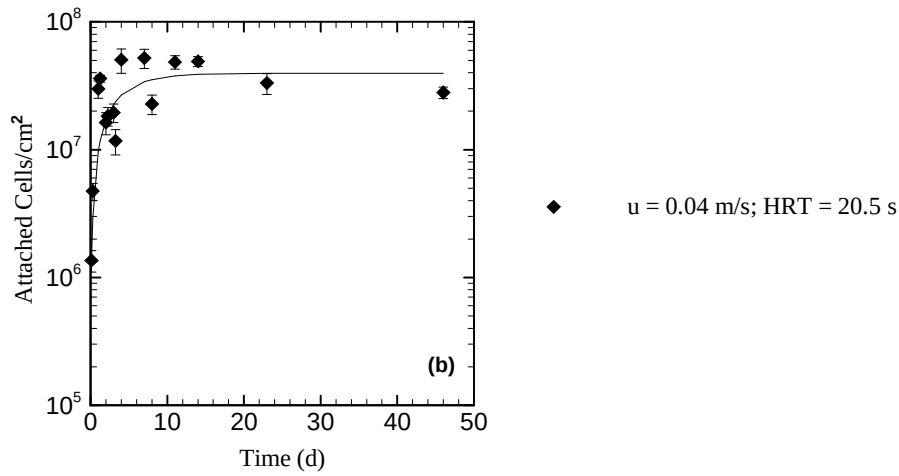


Figure 4.12 (cont.) - Biofilm cells per unit surface area of biofilms grown on PVC under the indicated flow velocity and residence time in: (a) - **Exp. 4.3** and (b) - **Exp. 4.4**. Eq. (2.21) is represented by the continuous line.

Using the values obtained for $(X_b)_{\max}$ and μ^*_{biofilm} , the asymptotic model (Eq. 2.21) is represented in Figure 4.11 and Figure 4.12 (continuous line) and gives a satisfactory fit of the experimental trends. This model assumes that steady state is achieved when there is a balance between biofilm removal and growth, which is

Table 4.3 - Specific cell growth rates at steady state (**Exp. 4.2**) for biofilms grown on PVC under turbulent flow ($u = 0.34 \text{ m/s}$; $\text{HRT} = 12.0 \text{ h}$).

Kinetic parameters	PVC
$(X_b)_{\max} \text{ (cell/cm}^2\text{)}$	$6.1 (\pm 2.5) \times 10^6$
$\Phi_p \text{ (cell/day)}$	$2.5 (\pm 1.4) \times 10^2$
$\mu^*_{\text{biofilm}} \text{ (day}^{-1}\text{)}$	0.15 ± 0.09
$\mu^*_{\text{bulk}} \text{ (day}^{-1}\text{)}$	1.8 ± 0.1
$\mu^*_{\text{overall}} \text{ (day}^{-1}\text{)}$	1.4 ± 0.2

just a conceptual approach because these events do not happen continuously and with the same extent. That is why some uncertainty exists that is not described by the model, as observed in Figure 4.11 and Figure 4.12.

It should also be emphasized that the average specific growth rates of cells in the biofilm were much lower than those in the microbial suspension (reactor bulk water), which agrees with the published literature in the last decade (Lappin-Scott and Costerton, 1995) where the metabolic state of attached cells was reported to be different from the one in suspended cultures. It should be noted that the specific growth rate in the liquid suspension is similar to the inverse of the hydraulic residence time, which is in accordance with the chemostat principles.

The specific growth rates for biofilms grown on PVC for different velocities and HRT are presented in Table 4.4. However, μ^*_{bulk} and μ^*_{overall} were not determined for **Exp 4.3** (lack of data of cells densities) neither for **Exp 4.4** ($X^* = X_{\text{in}}$ due to the low residence time).

For the same residence time (12.0 h), biofilms grown under lower shear stress (water velocities of 0.34, 0.24 and 0.04 m/s, **Exp. 4.2** and **4.3**) had a higher specific growth rate. Studies of the effect of shear stress on cell metabolism showed that different water velocities in contact with biofilms induced metabolic changes detected either by dehydrogenase activity and growth yield (Liu and Tay, 2001b) or gene and protein expression (Nauman *et al.*, 2007).

Table 4.4 - Specific cell growth rates at steady state (Exp. 4.3 and 4.4) for biofilms grown under different flow velocities and different residence time.

Kinetic parameters	Exp. 4.3 (u = 0.04 m/s; HRT = 12.0 h)	Exp. 4.3 (u = 0.24 m/s; HRT = 12.0 h)	Exp. 4.4 (u = 0.04 m/s; HRT = 20.5 s)
$(Xb)_{\text{max}}$ (cell/cm ²)	$5.3 (\pm 1.0) \times 10^7$	$2.2 (\pm 0.6) \times 10^7$	$4.0 (\pm 0.5) \times 10^7$
Φ_p (cell/day)	$3.5 (\pm 1.6) \times 10^3$	$1.2 (\pm 4.3) \times 10^2$	$3.0 (\pm) \times 10^3$
μ^*_{biofilm} (day ⁻¹)	0.24 ± 0.1	0.19 ± 0.05	0.28 ± 0.09

In the present work, a decrease in the shear stress resulted also in higher numbers of attached bacteria per square centimeter at steady state, probably due to a lower cell detachment effect. In laminar flow (0.04 m/s), the effect of decreasing the residence time from 12.0 h to 20.5 s increased the averaged biofilm growth rate, although with similar $(Xb)_{\text{max}}$. Probably bacteria were much more prone to attach to surfaces in the system with “harsher” conditions (*i.e.*, no water recirculation, lower residence

time) in order to enhance their survival opportunities. The effect of recirculating the reactor water changes several parameters relatively to the water coming directly from the tap (increases in temperature, organic carbon, bacteria densities...) which seem to affect the metabolic state of bacteria.

Several authors (Pedersen, 1990; Block *et al.*, 1993; Boe-Hansen *et al.*, 2002a; Boe-Hansen *et al.*, 2002b) reported lower growth rates for cells in drinking water biofilms, probably due to different shear stresses, lower temperatures of the water or chlorinated systems. The results of Boe-Hansen and co-workers (2002a) for specific growth rates in bulk water are around ten times higher than in biofilms, as in the present work. Other authors determined biofilm kinetics in wastewater and industrial cooling water systems and concluded that cell growth kinetics in biofilms was slower than in suspension (Wiesmann, 1994; Vieira and Melo, 1999). For example, specific growth rates of *Pseudomonas fluorescens* in suspended cultures are of the order of 10^{-1} h^{-1} (Robinson *et al.*, 1984), but the values found for the same species in a biofilm (Vieira and Melo, 1999) were 10 times lower. However, as opposed to the present case, these biofilms were quite thick (200 - 700 μm) and contained significant amounts of EPS that contributed to internal diffusional limitations and, possibly, to different metabolic states of the bacteria located along the depth of the biofilm matrix. In the present work, the biofilms were very thin and essentially composed by cell colonies; no EPS could be detected with the available methods. This fact may be related to the scarcity of nutrients in properly treated drinking water, as compared to wastewater or industrial cooling waters.

4.4 Conclusions

a) The study of biofilm formation in drinking water systems under the same flow velocity (Propella and Flow Cell reactors) and stagnant conditions (non-stirred Batch reactor) showed that the total cell counts per unit surface area were around one order of magnitude higher in the flow reactors than in the Batch reactor. Although the Flow Cell and Propella reactors have completely different designs, their performance was similar as regards bacterial accumulation on surfaces under the same flow velocity. However, the ratio of attached cells to suspended cells in the Propella was much higher than in the Flow Cell for the same velocity and residence time. This can be the result of higher temperature and Reynolds number in the Flow Cell, as well as of differences in the ratio volume/area and reactor design.

b) The water velocity affected the attached cells/suspended cells ratio in assays with the same residence time in the Flow Cell by presenting lower ratio values for higher velocities probably due to higher detachment rates. Increasing velocity from laminar to turbulent flow (0.04 -0.34 m/s) led to decreasing one order of magnitude the steady state values of wall cell accumulation (from 5.3×10^7 to 6.1×10^6 cell/cm²).

c) The residence time highly affected the suspended cell densities in the Flow Cell reactor although the attached cell numbers in biofilm were similar in steady state for the same velocity (two different residence times of 20.5 s and 12.0 h). This fact seems to indicate that the suspended cell density is not determinant in biofilm accumulation, but it should be emphasized that the temperature and probably the metabolic state of bacteria were very different in the two studied cases. This fact was supported by the higher fractions of CTC-reducing and of R₂A-cultivable bacteria in the reactor water.

d) In quasi-steady state in assays with 12.0 h of residence time, the metabolically active bacteria in biofilms were around 17 - 34 % of the total cells and 6 - 18 % was able to form colony units in R₂A, regardless the reactor geometry. Similarly, the percentages of suspended metabolically active and cultivable cells in the water inside the reactors were 35 - 50 % and 5 - 20 % of the total cells, respectively. Much lower fractions were observed for lower residence time (and consequently lower temperatures).

e) By applying a simple mathematical model to the whole growth curve of the biofilm, values of the specific cell growth in biofilms were obtained over time. In steady state, (with HRT = 12 h and $u = 0.34$ m/s), the specific cell growth rate in the biofilm, μ_{biofilm}^* , was substantially lower than in the water, μ_{bulk}^* , (0.15 day^{-1} versus 1.8 day^{-1}). The overall specific cell growth rate of the system, μ_{overall}^* (a weighed average value that represents the growth potential in the whole system, including suspended and attached biomass), was around $1.3\text{-}1.4 \text{ day}^{-1}$. Since the biofilms were thin and, therefore, no diffusional limitations are expected, it can be concluded that the metabolic state or the phenotype of the bacteria in the biofilm and in the water is different.

f) For the same HRT (12.0 h), increasing the velocity reduced the specific cell growth rate in the biofilm as well as the maximum total cell number per unit surface area. This also suggests possible metabolic changes related to differences in shear

stress over biofilms. For the same velocity (0.04 m/s), the averaged biofilm growth rate increased as HRT changed from 12.0 h to 20.5 s, although $(Xb)_{\max}$ was similar.

5. Biofilm Growth under Unsteady State Hydraulic Conditions

5.1 Introduction

The effects of unsteady-state flow (stagnancy and flushing periods) on the stability of drinking water systems were studied. Drinking water biofilms initially formed under continuous flow (turbulent or laminar) or under alternating flow regimes (stop/flow), were subjected to different stagnancy periods and/or different flow rates. The relationship between biofilm stability and bacteriological water quality was also studied for a simulated drinking water system subjected to increasing stagnancy periods. This study contributes to a better knowledge of the overall biomass growth processes in suspension and on the walls (accumulation and detachment) in drinking water distribution systems and, as such, will allow better water quality control, pipeline design and water distribution operation.

5.2 Experimental Methodology

In the present experimental work, a Flow Cell reactor with PVC coupons was connected directly to the drinking water tap without recirculation (see reactor

description in section 2.2.2). The operating parameters used for forming biofilms under different hydraulic regimes (under laminar and turbulent flow or in stagnant water) are presented in Table 5.1. Several assays were carried out in order to evaluate changes in bacterial numbers, either attached to the PVC surfaces or suspended in the water.

Table 5.1 - Operating conditions in Flow Cell reactor for the formation of biofilms under different hydraulic regimes.

Parameters	Hydraulic regimes		
	Stagnancy	Laminar Flow	Turbulent Flow
Flow rate (L/min)	0	1	6
u_{int} (m/s)	0	0.04	0.24
Reynolds number	0	1001	5797
τ_w (Pa)	0	0.01	1.10
HRT(s)	variable	20.5	3.0

Effect of stagnancy on biofilms formed under continuous steady flow. The biomass accumulation in the reactor during a stagnant period and the subsequent biofilm removal after flushing the system was assessed in **Exp. 5.1**. These assays aimed at simulating the use of water in houses from which people are absent during, for example, a week-end. Firstly, the biofilm was formed for 20 days under continuous flow either at 6 L/min (turbulent regime) or at 1 L/min (laminar regime). Secondly, a stagnant period was imposed for 3 days. After this period, all the reactor water was collected and finally the system was flushed at a flow rate of 6 L/min for 10 min (Figure 5.1). Biofilm and water samples were collected at three different instants: after 20 days of continuous flow (steady state), after the stagnancy period and after flushing the system. Both samples were analyzed for several physical, chemical and microbiological parameters such as total cells, R₂A-cultivable cell fraction and total organic carbon, according to the methods described chapter 2. In addition, the effect of stagnancy and the posterior flushing was evaluated for the metabolic activity of cells in the system where the biofilm was formed previously under turbulent flow.

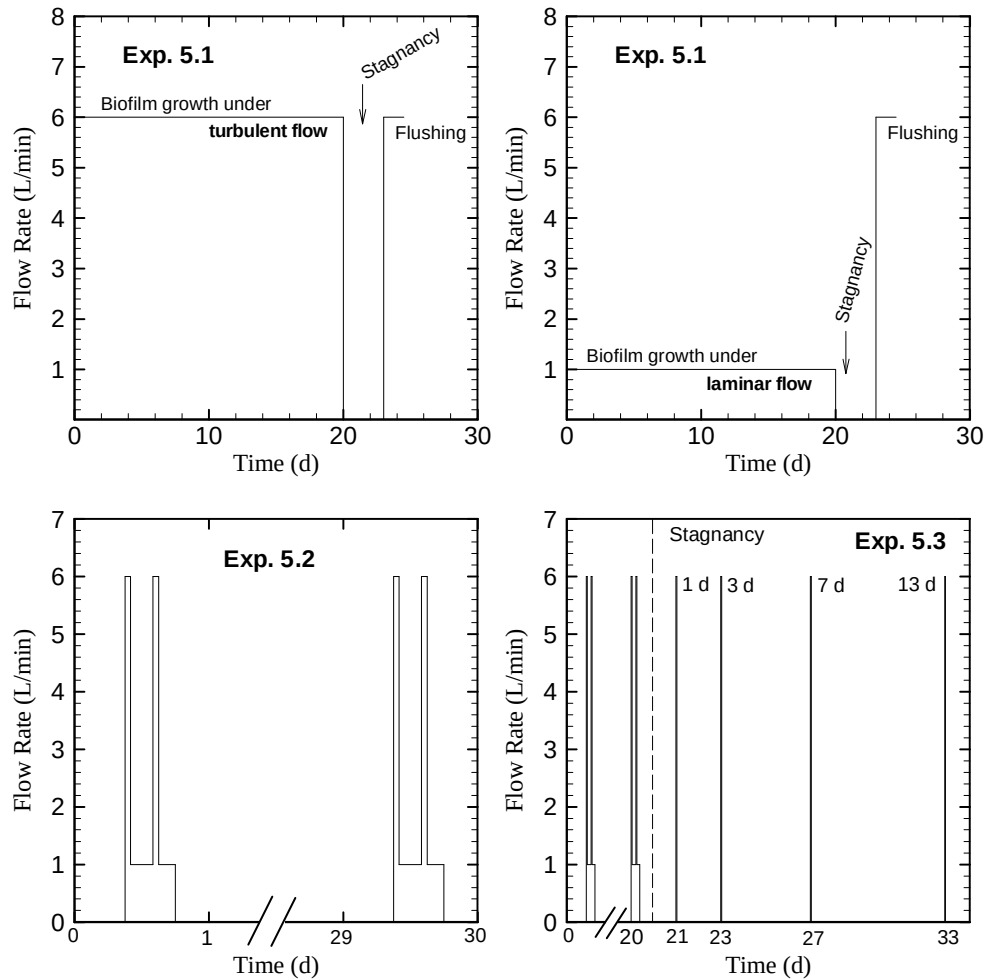


Figure 5.1 - Flow rate versus operation time for the different assays: **Exp. 5.1** - Biofilm was formed under turbulent or laminar flow and then the effect of stagnancy and biofilm removal at high flow rate was assayed; **Exp. 5.2** - The effect of alternating hydraulic regimes was assessed when biofilm was forming (from stagnancy to turbulent regime); **Exp. 5.3** - The effect of increasing the duration of the stagnancy periods on a biofilm previously formed under stop/flow regimes was evaluated.

Effect of unsteady hydraulic regimes on biofilm formation. In **Exp. 5.2**, the goal was to mimic situations where there is water consumption during day time, but not at night or week ends, such as it may happen in office buildings. Higher consumption (“rush hours”) in the beginning of the morning and the afternoon were also considered. For such purpose, biofilm accumulation under daily alternating flow regimes was evaluated during a 29 days assay. The daily hydraulic profile (Figure 5.1) was as follows: during the night (from 7 p.m. to 9 a.m. in the following morning) the tap was kept closed; from 9 a.m. to 10 a.m. the flow rate was increased up to 6 L/min (turbulent regime) for one hour (flushing of the system);

from 10 a.m. to 6 p.m. the flow rate was set to 1 L/min (laminar regime), except at the beginning of the afternoon (from 2 p.m. to 3 p.m.) when the flow rate was once more increases up to 6 L/min (turbulent flow). Biofilm samples were collected on days 4, 8, 15, 26, and 29, immediately after the stagnancy period (at 9 a.m.), and after flushing the system (at 10 a.m.). Biofilm and water samples were analyzed for the same parameters as those indicated in **Exp. 5.1**.

Effect of increasing the stagnancy periods on biofilm formed under unsteady state conditions. The relation between biofilm accumulation and the microbiological water quality under increasing stagnancy periods was evaluated in **Exp. 5.3** where the biofilm was previously grown for 20 days under variable hydraulic regimes similar to those described for **Exp. 5.2**, after which the tap was closed. After 1 day of stagnancy, two biofilm samples were collected immediately before and after flushing the system for 10 min at 6 L/min. Tap and reactor water samples were also collected before opening the tap ($t = 0$) and after flushing the system for 10 min. Then the system was kept under stagnancy, and samples were collected after 3, 7 and 13 days using flushing and sampling procedures described above (Figure 5.1). Biofilm and water samples were analyzed for the same parameters as those indicated in **Exp. 5.1**.

Effect of stagnancy on microbial composition. Two Flow Cells were operated simultaneously (**Exp. 5.4**), one under turbulent and other under laminar regime, according to the conditions described for **Exp. 5.1**. Biofilm samples were collected from each reactor during the continuous flow regime at day 14 (B61 and B11, from Flow Cells operating at 6 and 1 L/min, respectively) and at day 20 (B62 and B12, from Flow Cells operating at 6 and 1 L/min, respectively). Then both reactors were re-filled with sterilized bacteria-free drinking water and the flow was stopped. This stagnancy period lasted for 3 days and biofilm (B63 and B13, from Flow Cells previously operated at 6 and 1 L/min, respectively) and water samples (W6 and W1, from Flow Cells previously operated at 6 and 1 L/min, respectively) were collected. The microbial composition of each sample was determined through FISH and DGGE analysis, according with the methodologies described in sections 2.5.4 and 2.5.5.

5.3 Results and Discussion

5.3.1 Effect of Stagnancy and Flushing Periods on Biofilms Formed under Continuous Steady Flow

The effect of stagnancy on the biological characteristics of a simulated drinking water system was studied after the system had been operating under continuous flow in laminar or turbulent regimes (**Exp. 5.1**). Biomass accumulation adhered to the surface and in suspension was followed during the stagnant period and after flushing the system.

After 20 days of continuous turbulent flow, the steady state biofilm presented approximately 7×10^6 cell/cm² (Table 5.2), while the suspended total cell density (around $0.8 - 2 \times 10^5$ cell/mL) was in the same range of that found in tap water (Figure 5.2). In this biofilm, the fraction of R₂A-cultivable cells was 1.1 % (Table 5.2) while, in water samples, lower fractions were observed (< 0.4 %) (Table 5.3). This fact suggests that life in biofilm is truly improved particularly in systems like this one, with low contents of nutrients and with a disinfectant residual.

Table 5.2 - Total cell numbers per square centimeter and fraction of cultivable cells in biofilm samples collected under the indicated conditions in Exp. 5.1 (biofilm formed turbulent flow).

Conditions	Total cells/cm ²	Cultivable cell fraction (%)
After 20 days of biofilm growth under turbulent flow (Q = 6 L/min)	$7.4 (\pm 2.2) \times 10^6$	1.1 ± 0.4
After 3 days of stagnancy (Q = 0)	$1.1 (\pm 0.1) \times 10^7$	27.9 ± 4.7
After flushing at Q = 6 L/min for 10 min	$9.4 (\pm 1.7) \times 10^6$	7.1 ± 1.4

After the stagnancy period, the total cell number per square centimeter in the biofilm formed under turbulent flow increased 40 %, to values around 1×10^7 (Table 5.2), while in suspension the increase in the cell density was of 62.5 %. The temperature increase (from 14.2 to 18.5 °C) may be an important factor for these bacteria accumulation. The differences in the cell increase in the biofilm and in water were probably due to the fact that cell growth was higher in suspension than in biofilm, as observed in section 4.3.5 and also concluded elsewhere by other authors (Boe-Hansen *et al.*, 2002a).

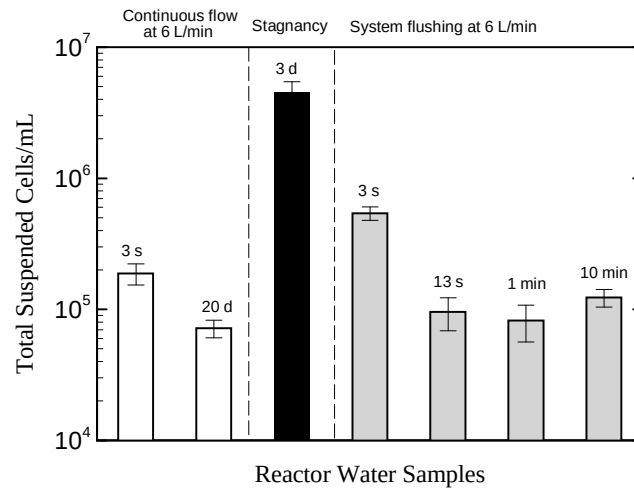


Figure 5.2 - Total cell density of reactor water obtained under the indicated conditions (**Exp. 5.1**). WHITE BARS: water samples collected when biofilm was formed during the first 20 days under **turbulent** flow; BLACK BAR: water samples collected after 3 days of stagnancy. GREY BARS: water samples collected during system flushing at 6 L/min.

Table 5.3 - Fraction of cultivable cells and total organic carbon in the reactor **water samples** obtained in **Exp. 5.1** with a biofilm formed under turbulent flow.

Conditions	Time	Cultivable cell fraction (%)	TOC (mg C/L)
Continuous turbulent flow at Q = 6 L/min	3 s	0.1 ± 0.1	2.9 ± 0.4
	20 day	0.4 ± 0.2	1.4 ± 0.1
Stagnancy (Q = 0)	3 day	64.0 ± 13.2	15.0 ± 1.2
Flushing at Q = 6 L/min	3 s	6.6 ± 1.4	2.7 ± 0.3
	13 s	3.7 ± 1.1	2.1 ± 0.4
	1 min	2.6 ± 1.5	2.6 ± 0.2
	10 min	2.1 ± 1.3	2.2 ± 0.2

In addition, during the stagnancy period the fraction of cultivable cells increased to 27.9 % in the biofilm (Table 5.2), and to 64.0 % in the water (Table 5.3). Again, the increase of both fractions was notoriously higher for cells in suspension, suggesting that cells grew faster in water than in biofilms, under conditions of non-flow. Interestingly, the stagnancy period promoted a very high increase in the cultivability capacity of the biofilm and suspended cells. Jorgensen *et al.* (1994) observed also high cultivability capacity as a response of *Pseudomonas fluorescens* to starvation. Additionally, nutrient-limited conditions induced resistance to

osmotic tension, oxidative stress and carbon limited culture conditions in *Pseudomonas putida* strain 2C8 (Gu and Mazzola, 2001).

After the stagnancy period, the empty Flow Cell was immediately flushed at a high flow rate (6 L/min) and after 10 min, the cells in biofilm and in water decreased to values similar ($p > 0.1$) to those found in steady state conditions (after 20 days of continuous flow) (Table 5.2 and Figure 5.2). These results suggest that almost all attached cells grown during the stagnancy period were removed during the flushing. The fact that the stagnancy period favored the increase of cultivable capacity of biofilm cells was confirmed because the detached biofilm layers, collected during the flushing, presented higher proportions of these cells when compared with those of steady state conditions (Table 5.3). The total organic carbon content of water followed the same trend observed for the cultivable fraction, *i.e.*, a pronounced increase was observed during the stagnant period.

In the reactor operated under laminar flow, the attached cells had the same behavior as those in the biofilm formed under turbulent flow (Table 5.4): biofilm total cells increased during stagnancy and then decreased during the flushing period.

However, in the water (Figure 5.3), a lower total cell density was measured after the stagnancy period and a lower decreasing rate of cell densities was observed during the flushing. These differences may be explained by the fact that biofilms formed under laminar flow are usually less resistant to removal as observed also elsewhere (Vieira *et al.*, 1993). The fractions of cultivable cells in the reactor water and the total organic carbon content (Table 5.5) presented the same evolution during the experiment as that observed in the system with the biofilm formed under turbulent flow.

*Table 5.4 - Total cell numbers per square centimeter and fraction of cultivable cells in **biofilm samples** collected under the indicated conditions in **Exp. 5.1** (biofilm formed laminar flow).*

Conditions	Total cells/cm ²	Cultivable cell fraction (%)
After 20 days of biofilm growth under turbulent flow at Q = 1 L/min	$7.8 (\pm 1.4) \times 10^6$	6.1 ± 1.3
After 3 days of stagnancy (Q = 0)	$2.6 (\pm 0.4) \times 10^7$	20.4 ± 4.6
After flushing at Q = 6 L/min for 10 min	$7.5 (\pm 3.3) \times 10^6$	2.4 ± 1.2

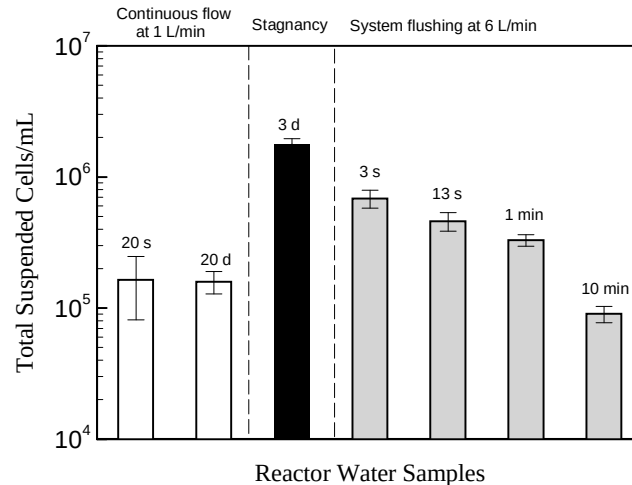


Figure 5.3 - Total cell density of reactor water obtained under the indicated conditions (**Exp. 5.1**). WHITE BARS: water samples collected when biofilm was formed during the first 20 days under **laminar** flow; BLACK BAR: water samples collected after 3 days of stagnancy. GREY BARS: water samples collected during the system flushing at 6 L/min.

A mechanistic explanation of the effects caused by the different hydraulic conditions may be given based on recent metabolic studies. Biofilm activity is highly affected by the detachment and growth. It has been reported that shear stress produces changes in bacterial metabolism in heterotrophic biofilms, favoring the catabolic activity. Given that the respiratory activity of cells is proportional to proton translocation across the cell membrane (Liu and Tay, 2001b; Liu *et al.*, 2003), higher shear stresses promote higher proton concentration outside the cell.

Table 5.5 - Fractions of cultivable cells and total organic carbon in the **reactor water samples** obtained in **Exp. 5.1** with a biofilm formed under laminar flow.

Conditions	Time	Cultivable cell fraction (%)	TOC (mg C/L)
Continuous laminar flow at Q = 1 L/min	20 s	N.D.	2.4 ± 0.1
	20 day	4.9	2.0 ± 0.5
Stagnancy (Q = 0)	3 day	93.6	14.8 ± 1.4
Flushing at Q = 6 L/min	3 s	8.6	3.4 ± 0.2
	13 s	8.1	2.6 ± 0.1
	1 min	4.5	2.5 ± 0.3
	10 min	4.2	2.2 ± 0.3

Under these conditions, bacterial surfaces present higher hydrophobicity, which in turn favors cell-to-cell and cell-surface interactions, *i.e.*, conditions that promote aggregation and biofilm formation (Tay *et al.*, 2000; Teo *et al.*, 2000). Therefore, when shear stress is reduced to zero a significant decrease in proton translocation may occur in the upper biofilm layer in contact with the flow and cells will tend to loose their adhesion strength. This effect would be much more visible in a biofilm which was structured under a higher shear stress than in one formed under lower flow rates. Therefore, in the present study, the biofilm initially subjected to turbulent flow may have suffered a more drastic reduction in the internal adhesion forces needed to keep its compact structure and, as a consequence, may have responded with a more substantial detachment of biomass to reactor water resulting in an increment of suspended cells of about 20 times after 3 days of stagnancy. On the other hand, the absence of shear stress (for 3 days in the present case), may have favored the shift of bacterial metabolism to anabolism, resulting in the observed overall increase of total cell densities (biofilm and suspended cells) in the system where the biofilm was grown under laminar regime. Probably the same situation occurred in the system with the biofilm grown under turbulent flow. However, due to the probable substantial detachment of biomass at the beginning of the stagnancy period, only the value of suspended cell density presented an important increase. The fact that the stagnancy period also improved metabolic activity and cultivability in R₂A-medium of the attached and suspended bacteria may be explained by an increase in the total organic carbon content, as observed for both systems (Table 5.3 and Table 5.5).

Considering that the bacterial species were similar (with similar overall growth) in both systems, the accumulation of total cells, attached or suspended, during the stagnancy period of 3 days and the subsequent biofilm detachment are presented in Table 5.6. These results show that, after 10 min of flushing, the total number of cells removed from the biofilm grown under turbulent flow was 20 times lower than in the laminar flow case. This means that the inner layers of the turbulent flow biofilm are much more resistant to detachment by shear stress than the ones in the laminar flow biofilm. This may be explained by the fact that the hydrodynamic conditions used in the flushing experiments are equal to the ones prevailing during biofilm growth under turbulent flow (6 L/min) and that the biofilm structure in this case is already adapted to such conditions.

In addition, the fractions of metabolically active cells were determined for biofilm and water samples for the system where the biofilm was formed under turbulent

Table 5.6 - Accumulation of total cells attached to the surface and in suspension during the stagnant period and after flushing obtained in Exp. 5.1 where biofilm was formed under turbulent or laminar flow.

Operating conditions	Total cell numbers (cells)	
	Turbulent regime (Q = 6L/min)	Laminar regime (Q = 1 L/min)
Biofilm cells after 20 days of continuous flow	$2.5 (\pm 0.7) \times 10^{10}$	$2.6 (\pm 0.5) \times 10^{10}$
Biofilm cells after 3 d of stagnancy	$8.7 (\pm 0.9) \times 10^9$	$6.1 (\pm 1.3) \times 10^{10}$
Suspended cells after 3 d of stagnancy	$1.0 (\pm 0.2) \times 10^{10}$	$3.6 (\pm 0.4) \times 10^9$
Biofilm cells removed after 10 min of flushing	$3.1 (\pm 7.4) \times 10^9$	$6.2 (\pm 0.2) \times 10^{10}$

flow (Table 5.7). After the 20 days of the continuous turbulent flow period, the fractions of metabolically active cells in biofilm and in suspension were around 13.5 and 7.5 %, respectively, while the corresponding cultivable fractions were lower. This difference between both fractions was similarly observed after flushing the system as was also obtained in the experiments presented in the chapters before. This fact may be explained by the inability of some cells of forming colonies in an agar medium although possessing metabolic activity (measured by the redox activity inside the cells). In opposite, the 3-days stagnancy period promoted a higher increase of cultivable fractions in the water phase and in biofilm (64.0 and 27.9 %) when compared to those of metabolically active cells (29.3 and 15.5 %, respectively). This opposite relation between both fractions may be attributed to a rapid nutrient decay during stagnancy due to the increase of cells during this period. The lack of nutrients may have lead to a decrease in the metabolic activity of the cells collected after 3 days of stagnancy, while maintaining their cultivability. The metabolic activity was measured by the capacity of CTC (redox potential -200 mV) catching electrons from the metabolic pathways. In truth, this will occur if electrons are transferred from cellular components with redox potentials lower than -200 mV. Enzymes involved in respiratory activity, as succinate and NADH dehydrogenases, are known to give electrons to CTC while oxidizing NADH to NAD^+ . Thus, the stagnancy may also promote a shift from catabolism (degradation of nutrients with income of energetic molecules) to anabolism (synthesis of cell constituents under energy expenses). However, it is not known if cellular components involved in anabolic reactions are also able to reduce CTC.

These results (higher fractions of cultivable cells than those of metabolically active) seemed at first glance to be opposite from other works (McDougald *et al.*, 1998; Gu and Mazzola, 2001; Ultee *et al.*, 2004) that described that cells underwent into a viable but not cultivable state (VBNC) as a physiological response of bacteria to enhance survival during environmental stress (*e. g.*, nutrient depletion, starvation, heat shock...). During the VBNC state, cells were still performing several metabolic processes at low levels, including nutrient transport, respiration rate, macromolecular biosynthesis and gene expression (Oliver, 2005). Cappelier *et al.* (2000) observed that protein biosynthesis was improved during starvation of *Campylobacter jejuni*. Moreover, these VBNC bacteria may resuscitate from this dormant survival state into an actively metabolizing state when conditions become favorable (increase of temperature and nutrients). According to this, the 7-days long period of R₂A-cultivability test may have resuscitated some “dormant” cells to form colonies. These “dormant” cells were performing a lower level of metabolism processes, and consequently, did not account for the metabolic active fraction. Indeed, the incubation period was chosen as the minimum time for maximizing the colonies number.

In the previous chapters, the fractions of metabolically active and the cultivable cells were generally higher in biofilms than in the water phase. In this case, an inversion was observed after de stagnancy period which may be attributed to the fact that cells in suspension grew faster (around 10 x) than in biofilms as observed in the previous chapter.

*Table 5.7 - Fractions of metabolically active cells in the **water** and **biofilm** samples obtained in **Exp. 5.1** with a biofilm formed under turbulent flow.*

Conditions	Time	Metabolically active cell fraction (%)	
		Biofilm Samples	Water samples
Continuous turbulent flow at Q = 6 L/min	3 s	---	8.8 ± 2.7
	20 day	13.5 ± 4.1	7.5 ± 2.0
Stagnancy (Q = 0)	3 day	15.5 ± 2.8	29.3 ± 5.1
Flushing at Q = 6 L/min	3 s	---	n.d.
	13 s	---	28.7 ± 4.7
	1 min	---	8.7 ± 1.5
	10 min	10.4 ± 3.8	11.2 ± 2.4

5.3.2 Effect of Unsteady Hydraulic Regimes on the Formation of Biofilm

An assay was performed to evaluate how biofilm accumulates on PVC over one month under alternating flow regimes (**Exp. 5.2**): in each 24 h period, the water flow was stopped during the night, while continuous flow, turbulent or laminar, was maintained during the day (Figure 5.1). In the morning of days 4, 8, 15 and 29, total and cultivable biofilm cells were enumerated before opening the tap and after flushing the system for 1 hour. The results obtained show that at each sampling day, the biofilm total and the cultivable cells per square centimeter were lower after flushing the system than the ones obtained before opening the tap (Figure 5.4). However, during the assay, the total cell number of biofilm kept increasing, indicating accumulation on the surface from day to day. The overall number of cultivable cells per square centimeter also increased until day 15, but kept constant from that day until the end of the assay. Given that the daily flushing of the system promoted a constant removal of total cells (around 2×10^6 cells/cm²), there was a decrease in the percentage of detached total cells over time (from 55 to 10 %). Also the fraction of cultivable cells in the biofilm decreased along the experiment from 15 to 1 %, suggesting that they are mainly located in the younger surface layers of the biofilm.

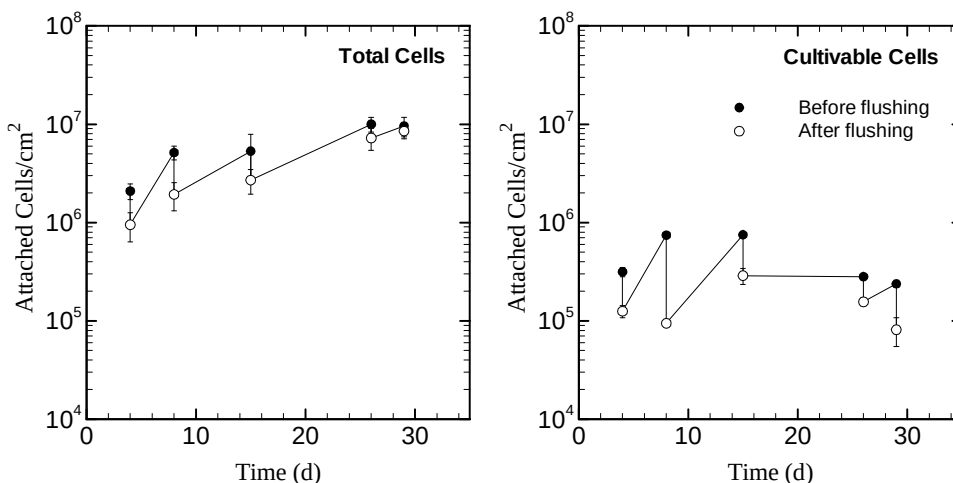


Figure 5.4 - Total and cultivable cells per unit surface area obtained for biofilms grown on PVC collected before and after opening the tap in the morning of each sampling day in **Exp. 5.2**.

Interestingly, after 15 days of operation, the values of total and cultivable cells per square centimeter were similar to those of steady state biofilms obtained in **Exp. 5.1**

indicating that the alternating flow / non-flow regimes do not affect the steady state accumulation in the wall.

When a drinking water biofilm builds up under successive alternating stop/flow conditions, the wall accumulation is lowered by periodically imposed detachment events, that released each time a constant amount of biofilm cells [$2.2 (\pm 0.9) \times 10^6$ cell/cm²]. These removed cells were those that accumulated during the period without shear stress and probably less adhered than the others that incorporated or grew in the biofilm during the flow conditions. The fraction of cultivable cells becomes smaller with biofilm age and depth because the settled bacteria in the older layers are dormant. The decrease of metabolic active bacteria with biofilm depth was also found out for thick biofilms in previous work (Zhang and Bishop, 1994b).

5.3.3 Effect of the Length of the Stagnancy Period on Biofilms Formed under Unsteady State Conditions

The effect of the length of the stagnancy period on cell accumulation, attached or suspended, was evaluated in **Exp. 5.3**. For this purpose, the reactor was kept under the unsteady state hydraulic regimes used in **Exp. 5.2** (Figure 5.1) for 20 days until biomass steady state was achieved. Subsequently, the tap was closed and samples were collected after stagnancy periods varying from 1 to 13 days. At each sampling day, total and cultivable cells of biofilm and water were enumerated before opening the tap and after flushing the system at 6 L/min. As observed in **Exp. 5.2**, the values of total and cultivable biofilm cells were higher before opening the tap than after flushing the system for 10 min (Figure 5.5). The loss of attached total cells due to flushing was compensated during the subsequent stagnancy period until 7 days of stagnancy. Thus, the total biofilm cells values were constant during this period.

However, on day 13 this value decreased probably due to the non-renewal of nutrients in prolonged periods of stagnancy. On the contrary, the loss of attached cultivable cells due to flushing was always compensated during the subsequent stagnancy period. Therefore, the cultivable cell fractions were constant and below 12 % until day 7, but increased to values around 30 - 40 %, due to the decrease of total biofilm cells on day 13.

The total cell numbers of water collected before opening the tap (stagnant water) increased with the duration of the stagnancy period (Figure 5.6), except when this period was prolonged to 13 days. In these stagnant water samples a high increase in

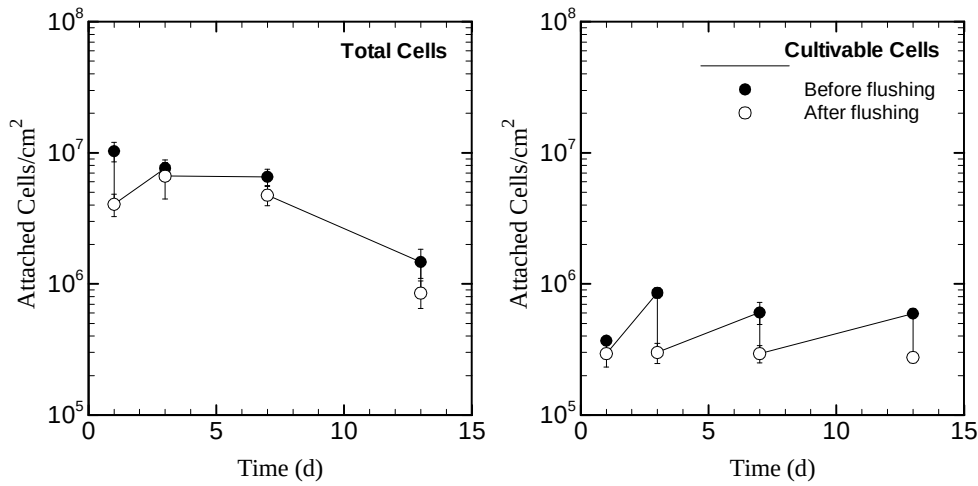


Figure 5.5 - Total and cultivable cells per unit surface area obtained for biofilms grown on PVC collected before and after opening the tap in the morning of each sampling day in **Exp. 5.3**.

the fraction of cultivable cells, from 40 to 60 %, and in the organic carbon content, from 7 to 27 mg C/L was observed (Figure 5.7 and Figure 5.8). As expected, the water samples collected after flushing the system presented values in total cells, cultivable fraction and carbon similar to those found in tap water (1×10^5 cells/mL, 5 % and 2 mg C/L, respectively).

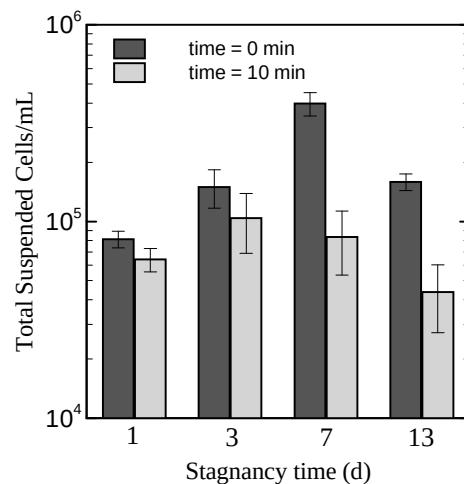


Figure 5.6 - Total suspended cell densities in reactor water samples before opening the tap ($t = 0$, stagnancy period) and after flushing the system for 10 min in **Exp. 5.3**.

Interestingly, while the density of biofilm cells until 7 days of stagnancy was similar to the one found after 3 days of stagnancy in **Exp. 5.1** (around 10^7 cells/cm², Table 5.2), the cell density values of stagnant water were 10 times lower (around

4×10^5 cells/mL in Exp. 5.3). Despite the high values, also the fraction of cultivable cells in stagnant water was lower (around 40 %) after 3 days of stagnancy (Figure 5.7) than in Exp. 5.1 (60 - 90 %). These results may indicate that, probably, the operating conditions imposed in **Exp. 5.1** and **Exp. 5.2** resulted in biofilms with different structures, and thus, systems with different distribution of attached and suspended cells.

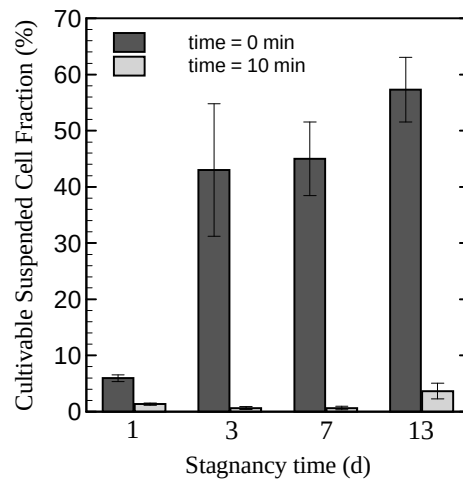


Figure 5.7 - Cultivable suspended cell fraction in reactor water samples observed after each stagnancy period, before ($t = 0$) and after flushing the system for 10 min in **Exp. 5.3**.

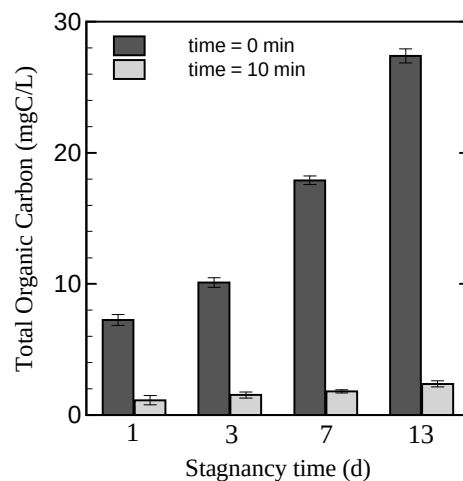


Figure 5.8 - Total organic carbon in reactor water samples after the indicated stagnancy period ($t = 0$) and after flushing the system for 10 min in **Exp. 5.3**.

Exp. 5.3 showed that the increase of duration of stagnancy periods led to different results when considering the total and the cultivable cell densities in the system. The total cell densities of attached and of suspended cells were, respectively,

maintained and increased only until 7 days of stagnancy, suggesting lack of nutrient renewal (*e.g.* growth factors, final electron acceptors) for longer periods. Eventually, these adverse conditions may lead to cell death with consequent release of organic carbon. On the other hand, the results obtained suggest that cultivability is directly related with the amount of organic carbon available in the system, since the number of cultivable cells was maintained or increased with the stagnancy time, respectively, in biofilms and in stagnant water.

In practical terms, these results indicate that short periods of stagnancy situations, such as those occurring in water reservoirs in buildings, promote the increase of total cells and the cultivable fraction, thereby degrading the biological quality of the water.

5.3.4 Effect of Stagnancy on Microbial Composition

5.3.4.1 FISH

The biofilm and water samples of **Exp. 5.4** were also analyzed by Fluorescent *in situ* Hybridization (FISH) as described in section 2.5.4. The bacteria were probed for the same phylogenetic groups that several authors used in samples from rivers (Cottrell and Kirchman, 2000; Araya *et al.*, 2003) and from drinking water systems (Kalmbach *et al.*, 2000; Batté *et al.*, 2003a; Horsch *et al.*, 2005; Jones *et al.*, 2007b). These groups were the following: *Alpha*-, *Beta*- and *Gamma*-subclasses of *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Nitrospirales* (nitrite-oxidizing bacteria) and *Nitrosomonadales* (ammonia-oxidizing bacteria). The results of FISH are presented as percentages of hybridized bacteria with each probe relatively to the total bacteria that were stained with DAPI.

The microbial compositions of biofilms and water samples collected in **Exp. 5.4** in the Flow Cell, where the biofilms were formed in turbulent and laminar flow regimes used in **Exp. 5.1**, are presented in Table 5.8 and Table 5.9, respectively. Biofilm samples collected during the continuous flow period presented similar microbial composition with predominance in *Betaproteobacteria* (33 to 37 %), followed by the subclasses *Alpha*- and *Gammaproteobacteria* with 10 - 16 % and 8 - 23 %. The microbial populations of drinking water samples presented similar compositions (section 3.3.4). Several authors observed by *in situ* hybridization, that *Betaproteobacteria* was one the most dominant groups in the microbial community in drinking water biofilms (Kalmbach *et al.*, 1997b; Schwartz *et al.*, 1998; Uhlmann *et al.*, 1998; Batté *et al.*, 2003a) and in bottled water (Loy *et al.*, 2005). Also,

Horsch *et al.* (2005) observed that the mature fouling layer in the filters in the drinking water treatment plant was dominated by bacteria of the *Alpha*- and *Beta*-subclasses of *Proteobacteria*, which was similar to the population structure of the raw surface water. Bacteria belonging to *Bacteroidetes* and *Actinobacteria* phyla accounted both with a maximum of 7 %. In agreement are the results of sequence analysis of 650 16S rRNA clones obtained from drinking water biofilms (Schmeisser *et al.*, 2003), that revealed the low percentages of *Actinobacteria* and *Bacteroidetes* relatively to *Proteobacteria* (in dominance). Nitrite oxidizing bacteria (NOB) and ammonia oxidizing bacteria (AOB) were also investigated and less than

Table 5.8 - Composition of the biofilm and water samples collected in **Exp. 5.4** from the Flow Cell at 6 L/min in terms of each targeted phylogenetic group.

Phylogenetic groups		Fractions of phylogenetic groups (%)			
		B61	B62	B63	W6
<i>Bacteria</i> domain subgroups	<i>Alphaproteobacteria</i>	12.4 ± 1.6	15.5 ± 2.4	11.5 ± 0.3	5.3 ± 3.4
	<i>Betaproteobacteria</i>	34.5 ± 3.4	32.7 ± 2.7	38.7 ± 4.5	41.1 ± 2.1
	<i>Gammaproteobacteria</i>	23.4 ± 2.7	15.3 ± 3.9	23.3 ± 1.8	32.3 ± 3.7
	<i>Bacteroidetes</i>	1.2 ± 0.1	3.4 ± 0.1	4.5 ± 1.2	5.3 ± 3.2
	<i>Actinobacteria</i>	1.2 ± 0.07	0.05 ± 0.02	2.1 ± 0.05	3.2 ± 2.1
	AOB (<i>Nitrosomonadales</i>)	2.3 ± 0.7	0.7 ± 0.04	1.8 ± 0.5	0.7 ± 0.2
	NOB (<i>Nitrospirales</i>)	1.1 ± 0.2	2.3 ± 0.4	1.4 ± 0.3	3.5 ± 0.8

AOB - ammonia oxidizing bacteria belonging to β - *Proteobacteria*; NOB - nitrite oxidizing bacteria.

Table 5.9 - Composition of the biofilm and water sample collected in **Exp. 5.4** from the Flow Cell at 1 L/min in terms of each targeted phylogenetic group.

Phylogenetic Groups		Fractions of phylogenetic groups (%)			
		B11	B12	B13	W1
<i>Bacteria</i> domain subgroups	<i>Alphaproteobacteria</i>	14.2 ± 3.7	10.1 ± 2.5	8.6 ± 1.9	10.7 ± 4.5
	<i>Betaproteobacteria</i>	37.3 ± 5.2	34.5 ± 2.7	40.4 ± 1.5	43.9 ± 5.3
	<i>Gammaproteobacteria</i>	8.5 ± 2.3	10.7 ± 1.6	31.8 ± 3.5	31.4 ± 2.2
	<i>Bacteroidetes</i>	5.4 ± 2.1	3.5 ± 0.1	4.1 ± 2.1	2.3 ± 1.2
	<i>Actinobacteria</i>	2.5 ± 0.01	4.1 ± 0.03	3.1 ± 0.1	2.7 ± 0.3
	AOB (<i>Nitrosomonadales</i>)	1.1 ± 0.04	0.8 ± 0.2	0.8 ± 0.1	0.5 ± 0.1
	NOB (<i>Nitrospirales</i>)	0.1 ± 0.04	0.1 ± 0.03	0.1 ± 0.03	0.1 ± 0.01

AOB - ammonia oxidizing bacteria belonging to β - *Proteobacteria*; NOB - nitrite oxidizing bacteria.

4 % was present, the AOB fraction being always higher than that of NOB. Several authors identified also these nitrification bacteria in biofilms and in suspended biomass in drinking water systems (Wolfe *et al.*, 1990; Regan *et al.*, 2002; Martiny *et al.*, 2003; Lipponen *et al.*, 2004; Hoefel *et al.*, 2005a; Martiny *et al.*, 2005).

During the stagnancy period, cells were allowed to grow probably at a higher room temperature (18.5 °C) than the one prevailing inside the distribution networks.

Microbial composition of biofilm samples after stagnancy was similar when compared to that of the adjacent water and presented higher percentages for the *Beta*- and *Gamma*-subclasses of *Proteobacteria*, *i.e.* 39 - 44 and 23 - 32 %, respectively. Thus, there was no preferential detachment of a particular phylogenetic group of bacteria to the bulk water during the stagnancy period

5.3.4.2 DGGE

Microbial composition was evaluated by DGGE in biofilm (B) and water (W) samples collected from reactor systems (**Exp 5.4**) during the continuous flow (turbulent and laminar) and after the stagnant period. Biofilm and water samples were processed according to the protocol described in section 2.5.5 and the corresponding electrophoresis gel is presented in Figure 5.9. The similarity matrices of the band profiles were obtained using Dice coefficient and the corresponding dendrograms (Figure 5.10) were built by UPGMA cluster analysis as described in section 2.7. These profiles also show the evolution of the bacterial communities with time and/or operating conditions.

Considering that each band corresponded to a single bacterial species, the DGGE profiles allowed the analysis of the total bacterial diversity in each sample (section 3.3.4), qualitative conclusions can be easily assessed just from observing the DGGE gel. For example, the profiles of bands of the biofilm samples collected during the continuous flow regime (B61, B62, B11 and B12) seemed to be less diverse and abundant than the samples collected after the stagnancy (that show higher number and more intense bands). After the stagnancy period, the intensity of bands of the biofilm profiles was different from the corresponding water samples, suggesting that the corresponding bacterial cells were differently released into the water. That difference seems more pronounced in W6/B63 (turbulent) than W1/B13 (laminar). This may be explained by effect of the previous flow regimes (turbulent and laminar) that produce biofilms with different detachments rates and compactness degrees.

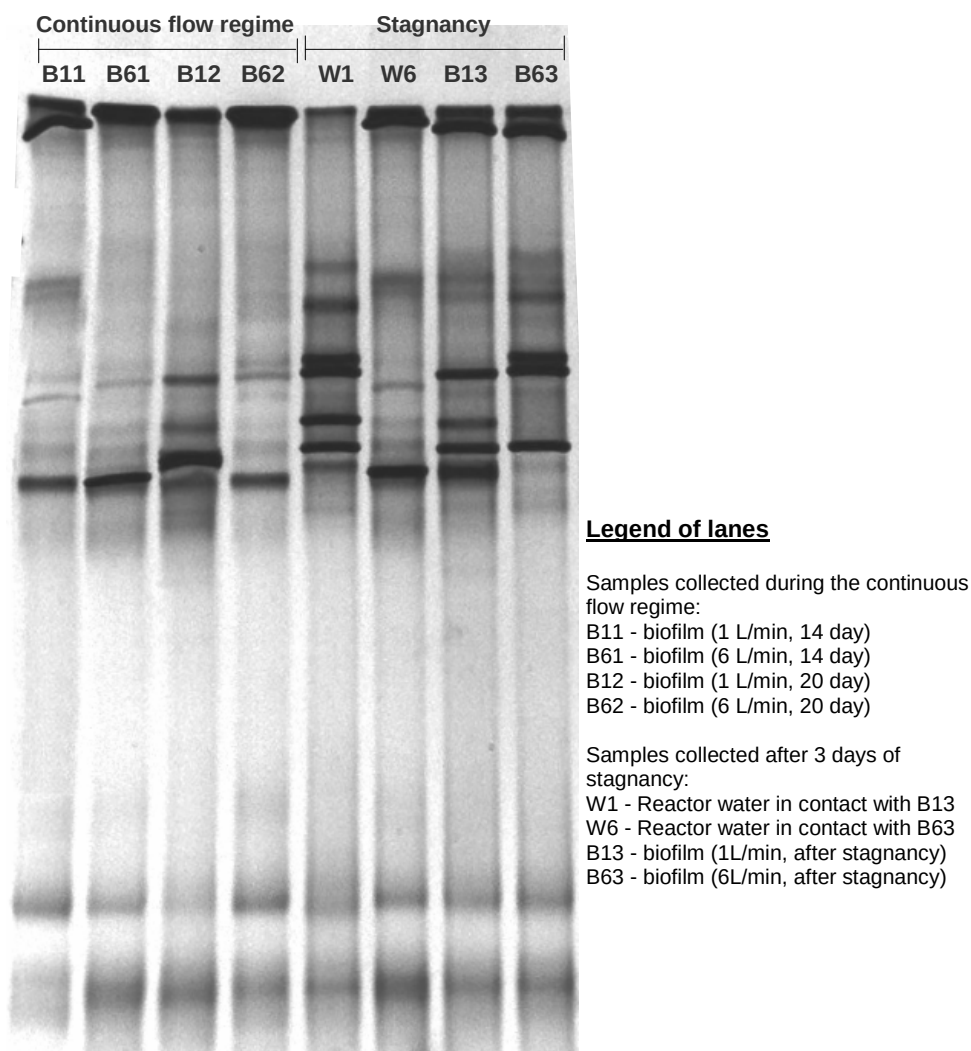


Figure 5.9 - DGGE patterns produced from 16S rDNA 180 bp fragments of biofilms and reactor water samples collected in Flow Cells operating at 1 and 6 L/min in *Exp. 5.4*. The legend of each lane is indicated above.

The DGGE gel profiles were transformed into a binary matrix of presence/absence (1/0) of each band. In this analysis, band intensities were not accounted as their relation was very questionable with initial bacterial composition. From this matrix, dendrograms of similarities (Figure 5.10a and b) were built and the conclusions were similar as those assessed by DGGE gel observation although more complete and quantitative. When comparing the similarities of the band profiles of the biofilm samples collected under continuous flow, on days 14 and 20, it was possible to observe some differences between the turbulent and the laminar regimes. Under

turbulent continuous flow, the biofilm bacterial communities were the most identical (Figure 5.10a), with DGGE profiles sharing 89 % of similarity indicating less bacterial modification than in laminar flow. Oppositely, under laminar flow the bacterial community on day 14 was less complex than that found on day 20 (Figure 5.9), which was confirmed by the lower similarity value (61 %) with all the other profiles in Figure 5.10b.

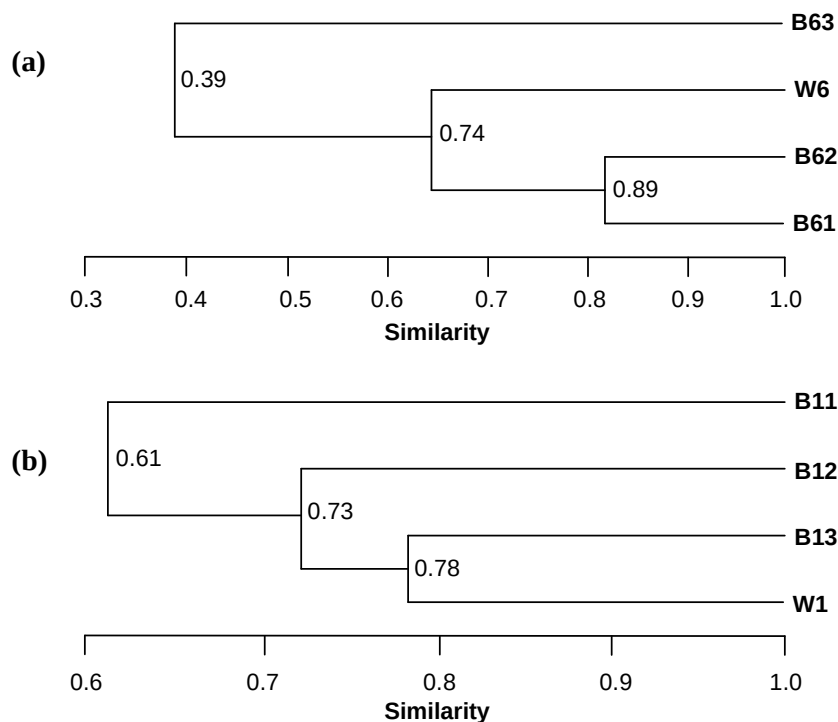


Figure 5.10 - Dendrograms representing the genetic similarity of the microbial community profile obtained by DGGE for the samples collected in the Flow Cell (**Exp. 5.4**) at: (a) - 6 and (b) - 1 L/min.

Comparing the biofilms immediately before and after the three days stagnancy, the variation in microbial populations was more pronounced in turbulent flow (43 % of similarity between B62 and B63) than in laminar flow (75 % of similarity between B12 and B13). This fact is in accordance to the fact that biofilms subjected to high shear stress will detach more substantially when the flow drops to zero.

After the stagnancy period, the DGGE patterns showed that several types of microorganisms present in the biofilm were released to the water. In fact, the DGGE profiles of the samples collected from the reactor with laminar flow showed that after stagnancy the bacterial composition of the biofilm (B13) and the water (W1) were similar (78 % of similarity). For the biofilm previously formed under

turbulent flow, the similarity of the bacterial profiles of biofilm (B63) collected after stagnancy was very low (39 %) when compared to all the other samples. These results suggest that due to the less compactness of laminar biofilms, more types of different organisms are released from the biofilms when compared with the more compact turbulent biofilms. In this late biofilm, cells present after 20 days of continuous flow were more prone to detach into the water than those grown during the stagnancy, which is accordance to the results obtained in Exp. 5.1, where a high increase of cells after stagnancy were observed.

A dendrogram (Figure 5.11) was also built to allow comparison between samples collected under turbulent and laminar flow in **Exp. 5.4**. Even with both systems being fed simultaneously from the same tap (thus, suspended microbial composition was similar), first biofilms were not that similar (60 % between B11 and B61). These dissimilarities tended to decrease with time: biofilms collected on day 20, B12 and B61, were 72 % similar. It is interesting to notice that biofilm samples after stagnancy, B13 and B63, were very similar (84 %). These facts suggest that effect of velocity on microbial composition was more pronounced in the initial stages of biofilm growth and that stagnancy preferentially promoted the growth of similar species in both biofilms.

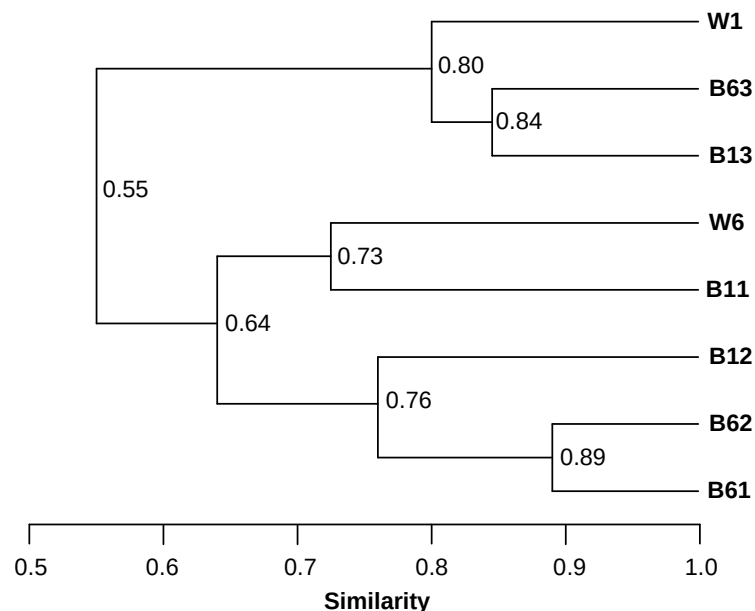


Figure 5.11 - Dendrogram representing the genetic similarity of the microbial community profile obtained by DGGE for the samples collected in the Flow Cell at 6 and 1 L/min (**Exp. 5.4**).

However the water samples (W6 and W1) had very different similarity coefficients (43 % and 78 %, respectively) when comparing with the adjacent biofilms. One possible reason is the fact that biofilms formed under laminar flow are less compact and cohesive and more porous, facilitating the bacteria exchange (detachment/attachment processes) and the variation in microbial population. On the other side, the biofilm formed under turbulent flow is more compacted and just a few microorganisms are released to the water phase. Consequently, higher dissimilarity was found between these two samples (W6 and B63) when compared to the others in laminar flow (W1 and B13).

It is interesting to notice that biofilm and water samples after stagnancy presented similar compositions with FISH for both experiments (turbulent and laminar flow regimes). However, DGGE analysis showed differences in the microbial composition of these systems because while FISH analysis distinguishes phylogenetic groups, DGGE distinguish species.

Although the conclusion by DGGE was different for the experiment under turbulent flow, they are not controversies. In FISH phylogenetic groups are identified while in DGGE each bacteria species originate different results (or bands).

5.4 Conclusions

The main conclusions are as follows:

- a) Stagnancy promoted biomass accumulation, in suspension and in biofilms, and caused a higher increase in the cultivability of cells than in their metabolically activity. This effect was more pronounced for the cells in the water than for those attached to the surface. The longer the stagnancy period, the higher the fraction of suspended cultivable cells, thereby degrading the water quality;
- b) Biofilms formed under turbulent or laminar flow behaved differently when subjected to prolonged periods of stagnancy: the former was more prone to release cells to the water phase and the latter kept higher numbers of attached cells when the water flow was stopped. When flushed, the decrease in cell number was slower in laminar biofilm;
- c) Daily alternating stop/flow operational procedures (unsteady state hydraulic regimes) contributed to the formation of a stable biofilm, containing decreasing fractions of cultivable cells over time;

d) Flushing systems that were previously kept under stagnant periods caused a release of a higher amount of biofilm cells into water. These cells might be formed (or attached) during the stagnancy period and probably were not strongly adhered. Given that these cells were in outer biofilm layers, where higher concentration of cells with metabolic activity and cultivability capacity were found, flushed biofilm had lower fractions of metabolically active and cultivable cells. However, these cells were undesirably contaminating the water;

e) Regardless the hydraulic regime, FISH analysis revealed that Betaproteobacteria was the most predominant phylogenetic group detected in all biofilms and water samples before and after stagnancy (constituting 32 - 44 % of total cells). Nitrite oxidizing bacteria (NOB) and ammonia oxidizing bacteria (AOB) were present in biofilm and water samples accounting for less than 4 %.

f) From DGGE analysis, different conclusions can be drawn for assays in turbulent and in laminar flows. Bacterial populations of biofilms formed in laminar flow were dissimilar in the first sample than the one formed in turbulent flow. This dissimilarity decreased with biofilm age, indicating that shear stress has more impact on the pioneer bacterial population rather than the steady state one. After a stagnancy period, the bacterial composition population of the biofilm formed under turbulent flow was very different from that of the adjacent water. Oppositely, these two types of samples were very similar (78 %) when the biofilm was formed previously in laminar flow. These facts are evidencing that the different biofilm structures were affecting the bacteria exchange to the water phase.

These conclusions can be of help in keeping potable water hygienic standards, mainly in cases where there are significant periods of stagnancy (from a few days to several months), such as in seasonal hotels, some hospital showers and water reservoirs in buildings, offices, weekend houses.

6. Material Effects on Biofilm Accumulation

6.1 Introduction

The effect of different adhesion materials on biofilm growth has been studied in order to find surfaces that can minimize its accumulation. However, the available research results are not conclusive and some are even contradictory, as summarized in section 1.7.5. Nowadays polymeric pipes have been preferred over the iron-based materials mainly due to their lower cost and easier application in drinking water networks. Nevertheless there are still some cases, where due to the system high pressure (usually in water pumping systems), ductile steel is necessary, sometimes as a concrete-lined pipe. Stainless steel is used as a security measure in pipes placed outside the walls in public buildings, and when a very high standard is required for water quality (*e.g.* water systems in dental surgeries). It has also been used indoor, in residential and office buildings, in recent years.

In this chapter, drinking water biofilms growing on different materials were followed in order to evaluate the growth potential on each adhesion surface. The tested polymeric materials were the most commonly used nowadays in water piping in drinking water systems (PVC, PEX, HDPE and PP). Stainless steel surfaces, with and without surface coating by implantation or deposition of different compounds (SiO_x, Excalibur, DLC and Ni-P-PTFE) were also evaluated. These materials had

very different surface properties and were chosen in order to evaluate possible correlations between their properties and biofilm accumulation.

Material surfaces were characterized by contact angles and roughness measurements and were visualized by EDIC/EF Microscopy and SEM/EDS. Biofilm accumulation was followed on the different materials under continuous flow and in stagnant water.

6.2 Experimental Methodology

The assays on the effect of the surface materials on biofilm accumulation were performed in the Flow Cell and in the Batch reactors, whose characteristics were presented in section 2.2. The Flow Cell units were connected in parallel, operating with recirculating tap water, which was also used to fill the Batch reactor. All the reactors were previously cleaned as described in section 2.2.5. The operating conditions of the assays with the different materials are presented in Table 6.1. Temperature was not controlled inside the reactors since the purpose was to observe the biofilm growth under the existing environmental conditions.

Surface Materials. Two groups of distinct materials were studied. The first group was formed by the polymeric materials PVC, HDPE, PEX and PP. The second group was composed by the metallic-based materials, such as the stainless steel (SS) and the surface modified SS (SiO_x, Excalibur, DLC and Ni-P-PTFE coatings). All these materials and their preparation were described in section 2.2.4.

Characterization of the surface materials. Prior to the assays, material surface characterization was performed by roughness and contact angles measurements (see procedures in sections 2.3.6 and 2.3.7). The surfaces were also observed by EDIC-EF Microscopy and Scanning Electron Microscopy coupled with Energy Dispersive x-Ray Spectroscopy, according to procedures described in sections 2.4.2 and 2.4.3, respectively.

Operating conditions. Several assays were performed to evaluate the effect of different surface materials on biofilm accumulation and on the kinetic parameters.

The Batch and the Flow Cell reactors were operated in **Exp. 6.1** and **Exp. 6.2** using the polymeric materials (PVC, HDPE, PEX and PP), respectively. Biofilm and reactor water samples were removed at predefined days from day 5 to 56. Several physical, chemical and microbiological parameters were measured, such as total, metabolically active and R₂A-cultivable cell numbers, total and dissolved organic

carbon, pH, total dissolved solids and temperature, according to the methodologies described in sections 2.3 and 2.4. Additionally, the effect of the continuous flow *versus* stagnant water (Batch reactor) regimes on biofilm growth was also assessed.

A third assay (**Exp. 6.3**) was performed in the Flow Cell, using PVC, SS and the other four surface modified stainless steels. From day 6 to 130, biofilm coupons and reactor water samples were collected and analyzed for the same parameters as determined in **Exp. 6.1** and **Exp. 6.2**. Biofilm samples formed during 3 months were carefully prepared for SEM observation and collected for FISH and DGGE analysis (see methods in sections 2.5.4 and 2.5.5).

Table 6.1 - Operating conditions in the biofilm monitoring reactors during the assays with the different material surfaces.

Parameters	Exp. 6.1	Exp. 6.2	Exp. 6.3	Exp. 6.4
Reactor	Batch	Flow Cell	Flow Cell	Flow Cell
Tested materials	PVC, PEX, HDPE, PP	PVC, PEX, HDPE, PP	PVC, SS, SiOx, Excalibur, DLC, Ni-P-PTFE	PVC and HDPE
u_{int} (m/s)	0	0.21	0.19	0.34
Q_{ext} (mL/h)	0	362.1	288	15.1
Reynolds No.	0	5000	4617	8293
τ_w (Pa)	0	0.80	0.80	1.91
HRT (h) or *(d)	*56	12.0	12.0	12.0
Duration (d)	56	56	130	30

The last experiment (**Exp. 6.4**) was performed only in the Flow Cell reactor, using PVC and HDPE as adhesion materials. The operating conditions are presented in Table 6.1. In the first days of this assay, biofilm and water samples were collected every 12 hours. After day 5, samples were collected at a rate of one per week. In this assay, the initial stages of biofilm growth on PVC and HDPE were observed in more detail and cell numbers obtained were used to determine biofilm growth rates and to compare the effect of the surface material on biofilm adhesion.

Principal Component Analysis. This statistical tool was performed with the experimental data obtained in **Exp. 6.1**, **Exp. 6.2** and **Exp. 6.3** according to procedures described in section 2.7 using the software SPSS (version 15.0, Rainbow Technologies). The main objective of this tool is to reduce the dimensionality of the experimental data consisting of several interrelated parameters, while retaining as much as possible of the data variation. This is

achieved by transforming values of the determined parameters into a new set of few variables, the principal components (PCs), which retain most of the variation present in all of the original parameters. These parameters were those measured for the different materials that are independent, namely, total cells/cm², fractions of metabolically active and cultivable cells present in biofilm, mean roughness, contact angle with water, negative component of surface free energy and total free energy of hydrophobicity. The whole output of this PCA tool is presented in section A.10.

6.3 Results and Discussion

6.3.1 Surface Energy and Hydrophobicity of Material Surfaces

Surface free energy was quantified after contact angles measurement for all materials with water (θ_w), formamide (θ_F) and α -bromonaphtalene (θ_B) and the results are presented in Table 6.2.

Hydrophobicity character may be evaluated by considering only the value of the contact angle with water (van Oss and Giese, 1995). Following this criterion, materials are hydrophobic if $\theta_w > 50^\circ$ and hydrophilic for lower values.

Table 6.2 - Contact angles of the surface materials measured with water (θ_w), formamide (θ_F) and α -bromonaphtalene (θ_B).

Materials	Contact angles ($^\circ$)		
	θ_w	θ_F	θ_B
PVC	69.3 $\pm$ 1.1	61.8 $\pm$ 2.0	60.6 $\pm$ 2.2
PEX	75.1 $\pm$ 3.2	57.8 $\pm$ 3.6	60.0 $\pm$ 3.1
HDPE	112.6 $\pm$ 3.1	59.8 $\pm$ 1.5	58.0 $\pm$ 2.2
PP	105.2 $\pm$ 2.8	69.8 $\pm$ 2.2	67.2 $\pm$ 0.5
SS	16.8 $\pm$ 1.6	42.7 $\pm$ 0.3	34.3 $\pm$ 1.6
SiOx	72.9 $\pm$ 3.0	63.7 $\pm$ 1.8	44.4 $\pm$ 3.5
Excalibur	116.6 $\pm$ 2.2	88.8 $\pm$ 1.6	77.7 $\pm$ 2.0
DLC	65.5 $\pm$ 1.7	50.4 $\pm$ 0.9	35.9 $\pm$ 4.7
Ni-P-PTFE	116.9 $\pm$ 1.4	100.0 $\pm$ 1.6	98.9 $\pm$ 2.2

All values are represented as average $\pm$ standard deviation calculated over ten independent measurements.

Considering the values presented in Table 6.2, stainless steel is the only hydrophilic surface while the others may be separated in two groups: low hydrophobic (PVC,

PEX, SiOx and DLC) and high hydrophobic materials (HDPE, PP, Excalibur and Ni-P-PTFE) ($p < 0.025$).

After measuring all the contact angles, the Lifshitz–van der Waals component (γ_S^{LW}), the electron-acceptor component (γ_S^+), the electron-donor component (γ_S^-), the polar component (γ_S^{AB}) and the total surface free energy (γ_S^{TOT}) were determined by applying the Acid-Base method (van Oss, 1997) and the results are presented in Table 6.3. This method describes very well the drop-surface equilibrium for all materials: the parameter RQ was always approaching 1.

The hydrophobicity is related to the surface free energy components: the increase of LW component indicates a lower polar surface and a high affinity to apolar liquids; the higher the AB component, the higher the amount of hydrating water and the hydrophilic character.

Among the polymeric materials, the affinity was always higher to apolar than to polar liquids, which is characteristic of hydrophobic surfaces. PVC and PEX distinguished from the HDPE and PP since the interaction with polar liquids was not null. The total surface free energy among these polymeric materials ranges slightly from 21 to 33 mJ.m^{-2} .

The surface energies of the modified materials were quite different. Although the γ_S^+ components were practically zero, the γ_S^- components presented values from

Table 6.3 - The components and the total surface free energy obtained for the tested surface materials according Acid-Base method and the non linear correlation coefficient.

Materials	Surface free energy (mJ.m^{-2})					RQ
	γ_S^{LW}	γ_S^+	γ_S^-	γ_S^{AB}	γ_S^{TOT}	
PVC	24.77 ± 1.10	0.66 ± 0.36	18.87 ± 2.17	7.05 ± 3.93	31.77 ± 3.43	1.000
PEX	24.99 ± 1.57	1.85 ± 1.03	9.48 ± 3.71	8.38 ± 5.70	33.37 ± 5.54	1.000
HDPE	25.91 ± 1.10	6.44 ± 1.05	0.00 ± 0.00	0.00 ± 0.00	25.91 ± 2.57	0.968
PP	21.35 ± 0.24	2.26 ± 0.75	0.00 ± 0.00	0.00 ± 0.00	21.35 ± 1.74	0.962
SS	36.08 ± 0.64	0.00 ± 0.00	72.67 ± 1.41	0.00 ± 0.00	36.08 ± 1.70	0.999
SiOx	32.64 ± 1.63	0.00 ± 0.00	16.31 ± 4.02	0.00 ± 0.00	32.71 ± 3.67	1.000
Excalibur	16.40 ± 0.93	0.34 ± 0.26	0.00 ± 0.00	0.00 ± 0.00	16.40 ± 1.35	0.963
DLC	36.37 ± 1.93	0.36 ± 0.23	16.16 ± 2.12	4.82 ± 3.15	41.16 ± 3.76	1.000
Ni-P-PTFE	7.94 ± 0.70	0.81 ± 0.39	0.19 ± 0.23	0.78 ± 1.02	8.74 ± 1.36	1.000

72.67 mJ.m⁻² to null for the different surface coatings. Thus, these materials had practically no ability to accept electrons, while the affinity to give them may be strong (as for SS surface) or null (as for Excalibur and Ni-P-PTFE coatings). The affinity to apolar liquids (LW) was much higher than the other relating interactions with polar liquids (AB) for which presented values around zero except for DLC (4.82 ± 3.15 mJ.m⁻²). Similar conclusion was observed when comparing the LW component with γ_s^- : the first one had the highest values, except for SS material. Therefore, γ_s^- apparently is not fit by it self to describe the hydrophobic character.

Azeredo (1998) stated that the hydrophobicity may also be evaluated from the surface free energy components. According to this author, if $\gamma_s^- \leq 25$ mJ.m⁻², the surface may be considered hydrophobic, independently from the value of the apolar component. If $25 < \gamma_s^- < 35$ mJ.m⁻², the hydrophobicity depends on the value of the apolar component: the surface is considered hydrophobic if $\gamma_s^{LW} \leq 45$ mJ.m⁻² or hydrophilic if $\gamma_s^{LW} \geq 46$ mJ.m⁻². The hydrophobicity is not dependent on the apolar component but higher values of this component indicate a higher hydrophobicity/hydrophilicity ratio. Following this criterion, the conclusions were similar to those based on the contact angle with the water: all surfaces are hydrophobic, except stainless steel.

The total free energy of hydrophobicity (ΔG^{TOT}) was determined according to the equations defined in section 2.3.6 and the obtained values for all surface materials (Table 6.4) were negative indicating again their hydrophobic characteristics (a high affinity to apolar liquids), except for stainless steel (66.61 ± 1.69 mJ.m⁻²).

In agreement with the conclusions above, HDPE, PP, Excalibur and Ni-P-PTFE were again more hydrophobic (lowest values of ΔG^{TOT}) than PVC, PEX, SiOx and DLC. Similar results were obtained by other authors (Santos *et al.*, 2004; Rosmaninho *et al.*, 2007) who worked with the same materials (SiOx, Ni-P-PTFE and stainless steel), except for DLC coating that was slightly hydrophilic.

6.3.2 Roughness Parameters

The surface roughness of the clean materials tested for the biofilm production potential was measured and the results are presented in Table 6.5. Ra is the mean roughness, Rq is the geometric mean roughness, Rz is the mean depth and the

Table 6.4 - The polar and apolar components and the total free energy of hydrophobicity for the surface materials.

Materials	Free energy of hydrophobicity (mJ.m ⁻²)		
	ΔG^{LW}	ΔG^{AB}	ΔG^{TOT}
PVC	-0.18 ± 0.13	-11.96 ± 10.36	-12.14 ± 10.36
PEX	-0.22 ± 0.21	-29.09 ± 18.35	-29.30 ± 18.35
HDPE	-0.35 ± 0.18	-50.74 ± 4.18	-51.10 ± 4.18
PP	0.00 ± 0.01	-71.63 ± 5.04	-71.63 ± 5.04
SS	-3.58 ± 0.29	70.19 ± 1.67	66.61 ± 1.69
SiOx	-2.18 ± 0.60	-20.43 ± 10.05	-22.61 ± 10.07
Excalibur	-0.77 ± 0.28	-90.22 ± 4.50	-90.99 ± 4.51
DLC	-3.71 ± 0.87	-18.33 ± 9.11	-22.04 ± 9.15
Ni-P-PTFE	-6.85 ± 0.92	-76.59 ± 7.19	-83.44 ± 7.25

Table 6.5 - Roughness parameters (Ra, Rq, Rz and Rmax) of the material surfaces tested for the biofilm production potential.

Materials	Roughness parameters (μm)			
	Ra	Rq	Rz	Rmax
PVC	0.15 ± 0.04	0.27 ± 0.08	1.68 ± 0.74	2.67 ± 1.54
PEX	0.80 ± 0.33	1.22 ± 0.27	5.41 ± 0.27	11.03 ± 3.49
HDPE	1.59 ± 0.41	2.18 ± 0.69	8.02 ± 1.50	13.76 ± 3.95
PP	0.64 ± 0.15	0.92 ± 0.20	4.47 ± 0.83	9.14 ± 2.44
SS	0.12 ± 0.02	0.22 ± 0.02	1.41 ± 0.10	1.75 ± 0.18
SiOx	0.08 ± 0.01	0.12 ± 0.02	0.66 ± 0.16	0.75 ± 0.15
Excalibur	0.85 ± 0.06	1.13 ± 0.08	5.53 ± 0.23	6.79 ± 0.66
DLC	0.07 ± 0.02	0.12 ± 0.02	0.68 ± 0.13	0.79 ± 0.12
Ni-P-PTFE	0.12 ± 0.02	0.20 ± 0.06	1.43 ± 0.46	1.65 ± 0.92

All values are represented as average $\pm$ standard deviation calculated over five independent measurements.

Rmax is the maximum depth measured within the sampling length (see section 2.3.7). None of these parameters distinguishes between peaks and valleys.

Comparing the four polymeric materials, the roughness increased in the following order: PVC < PEX $\approx$ PP < HDPE. Among these materials, the PVC may be considered the smoother surface with many small peaks/valleys and some

occasional higher depth variations. On the other hand, with the highest roughness, HDPE shows a more homogeneous and uniform peaks distribution at much higher values (lower than $R_{max} = 13.76 \pm 3.95 \mu\text{m}$).

For the modified surface materials and the stainless steel, the roughness increased in the order $\text{SiO}_x \approx \text{DLC} < \text{SS} \approx \text{Ni-P-PTFE} < \text{Excalibur}$.

6.3.3 EDIC+EF Microscopy

The materials surfaces were directly visualized by EDIC+EF Microscopy without any *prior* preparation and some of the pictures are presented in Figure 6.1 and others in section A.6.

Among the polymeric materials, PVC was the smoothest surface, although presenting several deep wells, while HPDE was the roughest surface.

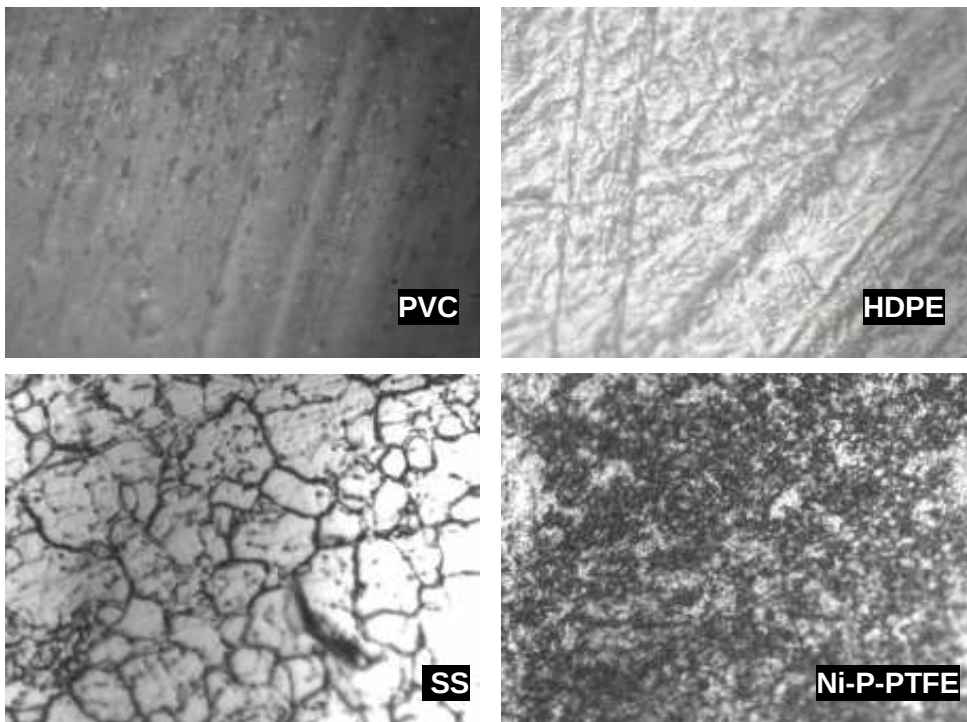


Figure 6.1 - Material surfaces observed under 1000 magnification with an EDIC-EF microscope (Area = $90.0 \times 67.2 \mu\text{m}^2$).

The stainless steel without and with surface modification (namely with SiO_x , Excalibur, DLC and Ni-P-PTFE) were also observed. Stainless steel was composed of compacted grains, surrounded in the limits of each by deep valleys. All the coatings were carefully prepared (G. Rizzo, University of Stuttgart) and

homogenous, covering the entire metallic matrix. Ni-P-PTFE was composed of a well distributed mixture of different components.

6.3.4 SEM/EDS Analysis

Scanning Electron Microscopy (SEM) coupled with elemental surface microanalysis of Energy Dispersive X-rays Spectroscopy (EDS) was also used to visualize the surfaces. These analyses had two major advantages over EDIC+EF, such as, the much higher magnifications and the composition of the surface. Material surfaces were previously prepared by CO₂ - critical point drying and then gold coating for the surfaces that were not electrical conductors (all except stainless steel). Surfaces were observed with 5000-15000 magnifications, by emitting x-rays with energy of 10-15 keV (the higher the energy, the higher the depth analyzed). Some of SEM pictures and EDS spectra are presented in Figure 6.2 and Figure 6.3 and others in section A.6 and A.9.

EDS microanalysis was performed while surfaces were being observed by SEM which may identify the elements on the first top layers (in the first several nanometers for 10 keV or in the micrometers order for 15 keV). The depth analysis depended on the elements composition and density. This microanalysis is a semi

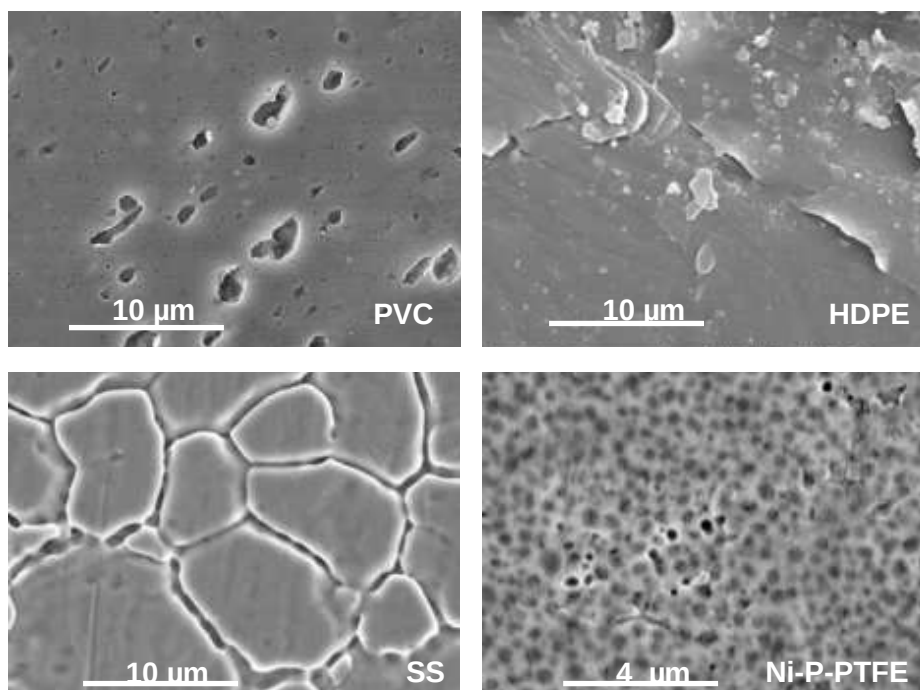


Figure 6.2 - Material surfaces visualized by SEM at 5000 to 15000 magnification, with a working distance of 15 mm and an x-ray energy of 10-15 kV.

quantitative technique since the elemental composition depends on several factors, such as: the location of elements on the analyzed volume, the type of elements present, the surface line, and the ability to emit photons that can cross over the surface and be captured by the detector, *etc.* Due to these limitations, EDS was more useful and gave more reliable results when the objective is to compare compositions of similar materials. This technique is based on the capture of the emitted photons by the detector that counts them and measures their energy. Knowing *a priori* the spectra of each released photons characteristic of each element, the elemental composition in weight fraction on each surface was quantified. EDS microanalysis can only quantify the elements with an atomic number between those of boron and uranium, the sensitivity of the analysis decreasing for elements with low atomic numbers (above potassium).

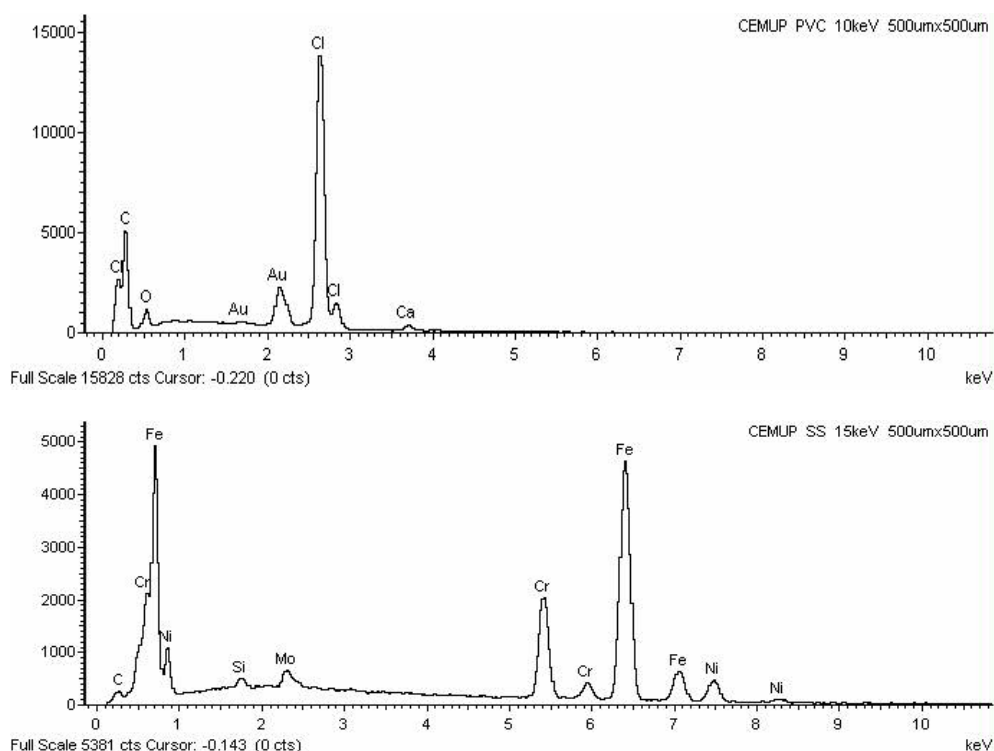


Figure 6.3 - Spectra of elements obtained by EDS microanalysis with 10-15 keV covering an area of $500 \times 500 \mu\text{m}^2$ on PVC and SS surfaces.

The composition was obtained without considering the presence of gold which was a part to the SEM/EDS surface coating and of oxygen since the stoichiometry of oxides was not known. The composition at the surface is presented in Table 6.6 for

polymeric materials and in Table 6.7 for surface modified materials and stainless steel. The SEM-EDS analyses complemented each other and were in accordance with the results from the roughness and the EDIC-EF analysis, although providing a greater amount of information.

The information from SEM coupled with the composition obtained from EDS (Table 6.6) indicated that PVC was the smoothest surface (with dispersed holes) and essentially composed by carbon and chloride with small amounts of calcium. The atomic C/Cl proportion was twice as higher (4.07) as the expected value for polyvinyl chloride. PEX (cross-linked polyethylene) and PP (polypropylene) surfaces were rough and composed mainly by carbon and other elements in low amounts, which were probably additives. The high density polyethylene (HDPE) had only carbon in the composition and was the roughest among the polymeric materials: both irregularities of several micrometers and smooth regions were observed.

Table 6.6 - Superficial composition of polymeric surfaces, obtained by energy dispersive x-rays spectroscopy analysis.

Element	Elemental composition (% wt.)			
	PVC	PEX	HDPE	PP
C	57.2 ± 1.1	94.7 ± 4.0	100.0 ± 4.4	97.4 ± 2.6
Na	n.d.	0.6 ± 0.1	n.d.	0.6 ± 0.1
Si	n.d.	1.2 ± 0.1	n.d.	0.9 ± 0.1
Cl	41.6 ± 1.0	1.4 ± 0.1	n.d.	1.2 ± 0.1
K	n.d.	1.4 ± 0.2	n.d.	n.d.
Ca	1.2 ± 0.1	0.7 ± 0.2	n.d.	n.d.

n.d. – not detected.

Stainless steel surface was composed by large flat regions surrounded by valleys corresponding to the grain limits composed by Fe, C, Ni, Cr, Si and Mo, a common composition of a stainless steel material. SiOx coating has a variable depth since depending on the area (A1 and A2, Table 6.7), EDIC pattern revealed the presence of Si with or without the contribution of SS composition. Excalibur and Ni-P-PTFE coatings were very homogeneous and thick films (of several micrometers) because the elements of SS were not detected by EDS. Santos *et al.* (2004) observed also that for thick coatings ($\geq 1 \mu\text{m}$) only the elements from the coating layers were measured. Excalibur, a fluoropolymeric coating, was formed by carbon and fluor.

The elements present in Ni-P-PTFE were, as expected in a coating, formed by nickel, phosphorous and polytetrafluoroethylene with the following amounts: 59.2 % of Ni, 6.6 % of P, 26 % of PTFE. SEM pictures make it difficult to observe the surface irregularities due to non-homogeneous composition of Ni-P-PTFE film and, consequently, the different grey colors.

Table 6.7 - Superficial composition of stainless steel and surface modified materials obtained by energy dispersive x-rays spectroscopy analysis.

Element	Elemental composition (% wt.)					
	SS	SiOx (area A1)	SiOx (area A2)	Excalibur	DLC	Ni-P- PTFE
C	1.2 ± 0.5	4.2 ± 0.5	n.d.	19.3 ± 0.5	89.1 ± 1.3	14.2 ± 0.9
F	n.d.	n.d.	n.d.	80.7 ± 0.6	n.d.	19.8 ± 0.4
Si	0.5 ± 0.1	3.3 ± 0.1	100.0 ± 0.1	n.d.	9.9 ± 0.2	n.d.
P	n.d.	n.d.	n.d.	n.d.	n.d.	6.6 ± 0.2
Cr	16.7 ± 0.3	18.5 ± 0.7	n.d.	n.d.	n.d.	n.d.
Fe	67.9 ± 0.7	67.4 ± 1.6	n.d.	n.d.	1.0 ± 0.4	n.d.
Ni	11.2 ± 0.4	6.6 ± 1.9	n.d.	n.d.	n.d.	59.2 ± 0.8
Mo	2.5 ± 0.2	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. – not detected.

6.3.5 Reactor Water - Effects of Hydraulic Regime

Reactor water samples were collected simultaneously with adhesion coupons during the studies of the biofilm growth potential on the different material surfaces (described section 6.2).

The physicochemical parameters of reactor water (Table 6.8) were constant and similar for all assays (**Exp. 6.1**, **Exp. 6.2**, **Exp. 6.3** and **Exp. 6.4**) approaching the values obtained for tap water, except for temperature and organic carbon content. The values of these parameters were higher than the corresponding ones in tap water, probably due to higher room temperature and cell accumulation during the experiment. Generally, the water inside the reactors was neutral (pH = 7.3 - 7.5) and, although higher than in tap water, presented low contents of the organic carbon (TOC = 3.2 - 3.9 mg C/L) and of dissolved solids (TDS = 158 - 172 mg/L).

Table 6.8 - Physicochemical parameters of reactor water samples collected during the assays of the biofilm growth potential on different materials.

	Exp. 6.1	Exp. 6.2	Exp. 6.3	Exp. 6.4
T (°C)	20.2 ± 1.0	21.0 ± 1.4	24.5 ± 1.9	22.3 ± 1.3
pH	7.4 ± 0.1	7.4 ± 0.1	7.3 ± 1.5	7.5 ± 0.2
O ₂ (mg O ₂ /L)	7.2 ± 1.1	6.2 ± 1.1	n.d.	ND
IC (mg C/L)	15.5 ± 0.1	16.2 ± 0.1	17.3 ± 0.1	16.7 ± 0.6
TOC (mg C/L)	3.2 ± 0.7	3.9 ± 1.1	3.3 ± 1.5	3.6 ± 1.8
DOC (mg C/L)	3.3 ± 0.7	3.8 ± 0.9	n.d.	3.4 ± 0.5
TDS (mg/L)	172 ± 7	165 ± 8	170 ± 29	158 ± 13

n.d. - not determined

The evolution of microbiological parameters (total, metabolically active and cultivable cell densities) was also followed for assays **Exp. 6.1**, **Exp. 6.2**, **Exp. 6.3** and **Exp. 6.4** (Figure 6.4). Independently from the assay and the reactor type, the cell densities increased during the first 5-10 days and then rapidly reached steady state values: around 1×10^7 total cells/mL inside the Flow Cell and 1×10^6 total cells/mL inside the Batch reactor. The higher values obtained in the former case

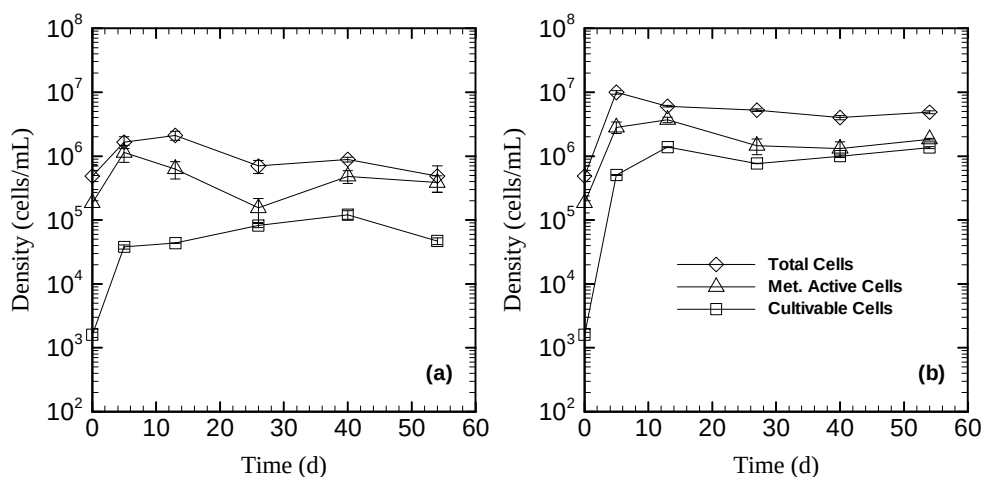


Figure 6.4 - Suspended cell densities in reactor water samples collected during the experiments to assess biofilm growth potential on different material surfaces in: (a) - **Exp. 6.1**, (b) - **Exp. 6.2**, (c) - **Exp. 6.3** and (d) - **Exp. 6.4**, defined in terms of total, metabolically active and cultivable cells.

may be attributed to the higher mean temperature and to cells removal from the surface in the continuous reactors. These facts may explain also the increase of the metabolically active and cultivable fractions in the reactor water (around 37.5 - 55.3 % and 9.5 - 30.2 %, respectively) when compared to the tap water values.

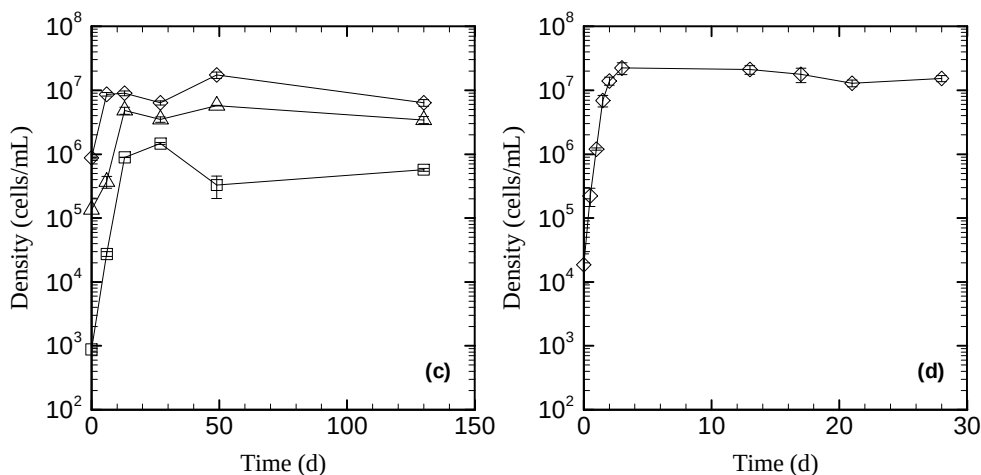


Figure 6.4 (cont.) - Suspended cell densities in reactor water samples collected during the experiments to assess biofilm growth potential on different material surfaces in: (a) - Exp.6.1, (b) - Exp. 6.2, (c) - Exp.6.3 and (d) - Exp. 6.4, defined in terms of total, metabolically active and cultivable cells.

6.3.6 Effects of Polymeric Materials on Biofilm

Biofilms were grown on common polymeric surfaces (PVC, PEX, HDPE and PP) in the Batch (**Exp. 6.1**) and the Flow Cell reactors (**Exp. 6.2**), and the attached cells per square centimeter were obtained in terms of total, metabolically active and cultivable numbers during the experiments.

In the Batch reactor, the results from the biofilms collected from day 5 to day 54 were constant over time for the total, metabolically active and cultivable cells. Figure 6.5 represents of biofilm grown on PVC. Plots of cells versus time for other materials are presented in section A.7.

Steady state values were determined (Table 6.9) indicating that $5.8 - 9.1 \times 10^6$ cells were colonizing each square centimeter of the material surface. Although these results were included in a narrow range, PVC, PEX and PP were statistically different ($p < 0.00175$), while HDPE and PP were equal ($p = 0.4175$).

The mean fractions of metabolically active cells were generally higher than the cultivable fraction and were in the range of 20.6 - 32.8 % for the CTC-reducing cells and 10.8 - 14.2 % for cultivable cells. HDPE and PP presented also similar values for the fractions of metabolically active and cultivable cells ($p > 0.2025$).

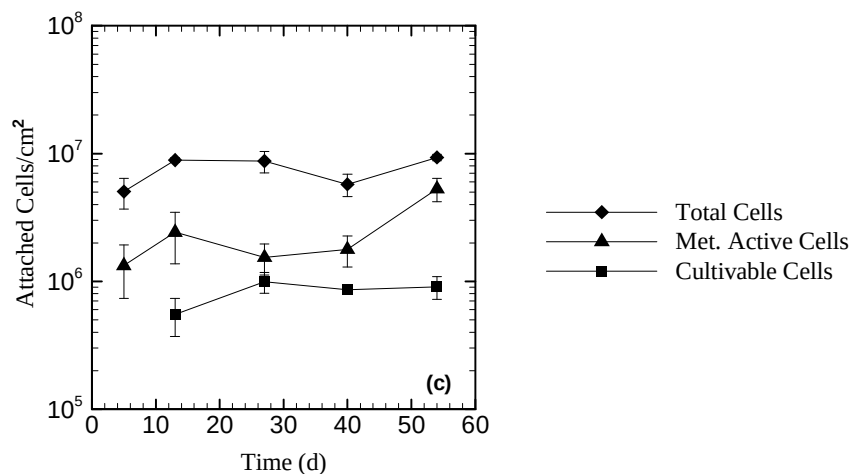


Figure 6.5 - Attached cells per unit surface area (total, metabolically active and cultivable cells) for biofilms grown on PVC in the Batch reactor (**Exp. 6.1**).

Table 6.9 - Total, metabolically active and cultivable cells in biofilms grown on polymeric surfaces in steady state in Batch reactor (**Exp. 6.1**).

Materials	Total Cells/cm ²	% of Metabolic Active Cells	% of R ₂ A Cultivable Cells
PVC	$7.5 (\pm 0.5) \times 10^6$	32.8 ± 5.1	11.0 ± 1.3
PEX	$9.1 (\pm 0.4) \times 10^6$	25.3 ± 3.2	14.2 ± 1.6
HDPE	$5.8 (\pm 0.4) \times 10^6$	20.6 ± 2.6	10.8 ± 2.9
PP	$6.0 (\pm 0.5) \times 10^6$	20.8 ± 3.2	12.8 ± 1.2

Biofilm growth potential was also evaluated in a Flow Cell reactor using the same polymeric materials and operation time. The results of biofilm accumulation over time were quite similar to those observed in Batch reactor. As observed previously in the Batch reactor, the metabolically active fractions were generally higher than the cultivable ones. Steady state values were obtained in the range of $2.6 - 8.7 \times 10^7$ of total attached cells/cm² (Table 6.10). These values were statistically different ($p < 0.01$) and increased in the following order of surface materials: PVC < PEX < PP < HDPE. The fractions of metabolically active and cultivable cells were in the range of 18.9 - 31.1 % and 10.6 - 16.6 % respectively.

Comparing the results in the two reactors (stagnant versus flow systems), the average total cell numbers per unit surface area in quasi-steady state were lower in the Batch reactor than in the flow reactor. For both reactors, it can be said that the quasi-steady state biofilm amount was not greatly affected by the surface material

Table 6.10 - Total, metabolically active and cultivable cells in biofilms grown on polymeric surfaces in steady state in the Flow Cell (**Exp.6.2**).

Materials	Total Cells/cm ²	% of Metabolic Active Cells	% of R ₂ A Cultivable Cells
PVC	$2.6 (\pm 0.1) \times 10^7$	24.2 ± 3.5	13.2 ± 2.2
PEX	$3.5 (\pm 0.2) \times 10^7$	31.1 ± 4.1	14.5 ± 1.1
HDPE	$8.7 (\pm 0.2) \times 10^7$	24.3 ± 2.3	10.6 ± 1.5
PP	$4.9 (\pm 0.3) \times 10^7$	18.9 ± 2.2	16.6 ± 1.3

since the results were contained in a narrow range (the same log scale unit); the same occurred with the biofilm relative composition in terms of metabolically active *versus* total cells, indicating that the active fraction of biofilms did not depend that much on the surface composition.

Some researchers (Clark *et al.*, 1994; van der Kooij and Veenendaal, 2001) observed that PE supports biofilm formation in a higher degree than PVC, while others (Kerr *et al.*, 1999; Niquette *et al.*, 2000; Zacheus *et al.*, 2000; Schwartz *et al.*, 2003b; Wingender and Flemming, 2004; Obst and Schwartz, 2007) concluded that there was no significant difference in the colonization of the investigated materials (stainless steel, PVC, PEX and PE), in some cases after decades of operation.

The analysis of data (with Principal Components Analysis, section A.10) from experiments in stagnant water regime (**Exp. 6.1**) and in flow system (**Exp. 6.2**) showed completely different dependencies indicating different effects and behaviors for each hydrodynamic regime. In **Exp. 6.1**, the materials that presented more similar behavior were PP and HDPE (with different roughnesses), and as a consequence, total attached cells per unit surface area were not affected by roughness. In **Exp. 6.2** the most similar materials were PVC and PEX, and a good tendency correlation between rugosity and cell adhesion (see Figure 6.6) was observed when the water was flowing in the Flow Cell reactor ($r = 0.959$, $p = 0.0404$).

For both reactors, the total free energy of hydrophobicity did not correlated with the total cell numbers accumulated in the biofilm. Surprisingly, a very good tendency ($r = -0.992$, $p = 0.0797$, Figure 6.7) was obtained in the Flow Cell system (**Exp. 6.2**) considering only materials with more close Ra values (excluding HDPE). This

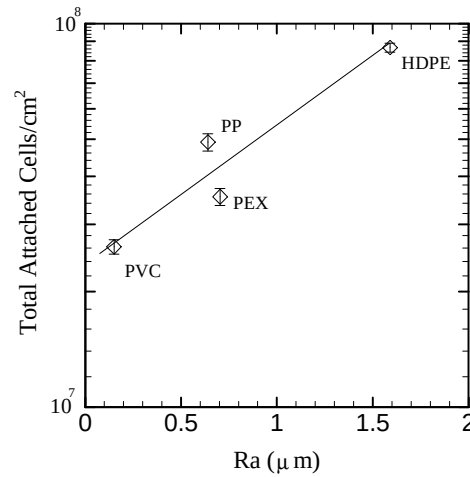


Figure 6.6 - Total attached cells per unit surface area versus the mean roughness for steady state biofilms grown on polymeric materials in the Flow Cell reactor (**Exp. 6.2**).

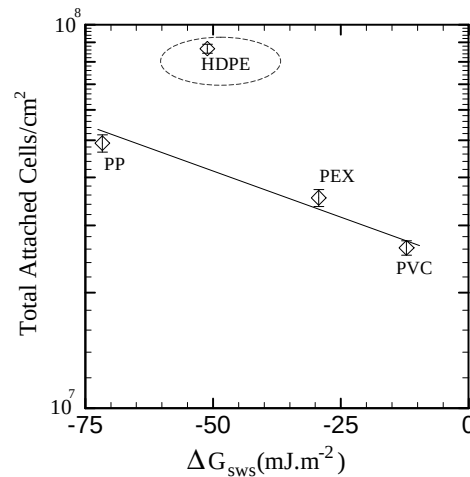


Figure 6.7 - Total attached cells per unit surface area versus the free energy of hydrophobicity for steady state biofilms grown on polymeric materials in the Flow Cell reactor (**Exp. 6.2**).

phenomenon (the increase in the biofilm accumulation with the hydrophobicity) was already observed by other authors (Teixeira and Oliveira, 1999; Pereira *et al.*, 2000; Cerca *et al.*, 2005; Pereni *et al.*, 2006) for both hydrophobic and hydrophilic materials. Oppositively Simões *et al.* (2007) concluded that the physical chemistry of the surfaces can not explain by itself the adhesion of isolated drinking water bacteria to several surfaces. Another important factor is the water composition and the interactions with the bacteria and the surface. For example, the presence of divalent cations, such as Ca^{2+} , Mg^{2+} and Fe^{2+} favored the adhesion of cells to more negatively charged polymeric materials (Teixeira and Oliveira, 2000).

The fact of not finding any correlation of total attached cells/cm² with mean roughness and total energy of hydrophobicity in **Exp. 6.1** may be attributed to the long operating time, low detachment phenomena, which probably reduced the effect of roughness on biofilm accumulation in stagnant systems.

6.3.7 Effect of Modified Surface Materials on Biofilm

New developed surface coatings on stainless steel were evaluated for the biofilm growth potential in Flow Cell reactor in **Exp. 6.3**. These coatings modified the surface in terms of composition, roughness and hydrophobicity parameters which were already presented in the previous sections.

Biofilm accumulation was followed for 130 days and analyzed for the total, metabolically active and cultivable cells per unit surface area. The patterns of biofilm accumulation on these materials followed the same trend the one observed in Batch reactor (Figure 6.5) and were presented in Figure A.5. An increase of the number of biofilm cells was observed until day 10 reaching then a quasi-steady state phase; also, the fraction of metabolically active was also always higher than the fraction of cultivable cells.

Steady state values of biofilm accumulation are presented in Table 6.11. Around $0.6 - 7.6 \times 10^7$ cells/cm² were attached to the surface of each material in the following increasing order: DLC < SS < Excalibur < SiOx < Ni-P-PTFE ($p < 0.0132$). In these biofilms, the percentages of metabolically active and cultivable cells were very different for the different materials, presenting values in the range of 2.4 - 47.4 % and of 0.9 - 10.5 %, respectively.

*Table 6.11 - Total cells per unit surface area and the fractions of metabolically active and cultivable cells in biofilms grown on stainless steel with and without surface coatings (SiOx, Excalibur, DLC and Ni-P-PTFE) in steady state in Flow Cell reactor (**Exp. 6.3**).*

Materials	Total Cells/cm ²	% of Metabolic Active Cells	% of R ₂ A Cultivable Cells
SS	$1.6 (\pm 0.1) \times 10^7$	18.1 ± 2.9	2.8 ± 0.3
SiOx	$5.6 (\pm 0.2) \times 10^7$	2.4 ± 0.7	2.4 ± 0.3
Excalibur	$2.1 (\pm 0.2) \times 10^7$	17.0 ± 4.3	4.4 ± 0.6
DLC	$6.1 (\pm 0.6) \times 10^6$	47.4 ± 17.2	10.5 ± 1.3
Ni-P-PTFE	$7.6 (\pm 0.6) \times 10^7$	6.7 ± 1.5	0.9 ± 0.1

After applying Principal Component Analysis for this experimental data, no correlations were observed with the roughness and hydrophobicity, probably due to the interference of a non-measured parameter (*e.g.* chemical composition). SiO_x and Ni-P-PTFE were considered the materials with the most similar parameters, such as total attached cells/cm², mean roughnesses and fraction of metabolically active and cultivable cells (Figure A.12). The other materials were apart mainly due to: the high positive γ_s^- value of SS; the low total cells/cm² and high fractions of metabolically active and cultivable cells attached onto DLC coating; and the low γ_s^- value and high mean roughness of Excalibur. As observed by other authors, the chemical composition of the adhesion material may affect directly the biofilm accumulation. For example, Lopes *et al.* (2005) observed that the presence of nickel in steel samples promoted cell adhesion to the surfaces. Other authors (Fang *et al.*, 2002; Lawrence *et al.*, 2004) observed that several elements in solution (Ni, Cr and Cu) affected the river water biofilm amount, as well as its microbial community diversity and function (*e.g.* Ni reduced the proportion of exopolymeric substances).

Zhao and Liu (2006) observed that *Escherichia coli* attachment to stainless steel, during 20 hours, could be reduced when Ni-P-PTFE was deposited on the surface with low amounts of PTFE (0.55 % wt.), otherwise the effect was much less noticeable. The coating tested in this work had much higher amount of PTFE (26 % wt.) and the operating time was also much higher. The operating time may reduce the material effect because biofilm grows and becomes thicker with the new forming layers far from those attached to the surface. For example, biofilms grew faster in PE than in copper pipes, but such differences could not be detected in older piping systems (Lehtola *et al.*, 2004; Lehtola *et al.*, 2005).

6.3.8 Surface Analysis of Biofilms

Drinking water biofilms were grown on several materials during 3 months in the Flow Cell reactor. These samples were collected in duplicate for each material surface and prepared immediately for subsequent SEM observation.

The structures of biofilm samples are extremely difficult to preserve for SEM observation as a consequence of their high water content (around 97 %) that must be completely purged before SEM visualization (Sutherland, 2001b). SEM is operated in vacuum that is degraded in the presence of water or any other liquids. Therefore, the water hydrating the biofilm matrix was firstly replaced by ethanol which was afterwards completely removed by drying at room temperature followed

by CO₂ replacement at critical point (31°C + 73 atm). The dehydration process may collapse the biofilm, giving the incorrect impression that mature biofilms are confluent (Law *et al.*, 2001).

Glutaraldehyde was used before replacing the water by ethanol in order to avoid the release of the cell contents and therefore protecting the bacteria against deformation due to dehydration. Apart from binding to cell membranes, glutaraldehyde may also dissolve the EPS as observed by other authors (Azeredo, 1998; Azeredo *et al.*, 1999). Therefore, using low amount of this reactant will reduce extensively the EPS dissolution and still protect bacteria from collapsing or lysing and minimize the damage on the biofilm structure.

Some pictures of biofilms grown on the different materials are presented in Figure 6.8. The squares indicate the area (A) where the micro-analysis by Energy Dispersive X-Ray Spectroscopy was performed. The spectra corresponding to this analysis is presented in section A.9 and the corresponding results of surface composition are presented in Table 6.12 and Table 6.13.

Descriptions of biofilms and their elemental compositions based on these analyses should be related with the original biofilm with care/precautions. The elemental composition on the top layers may be different from the real values because solvent replacement of water removes EPS and may reduce the concentration of elements bound within the biofilm (Little *et al.*, 1991). Nevertheless, the differences in the contents between the biofilm and surface may give valuable information about the attached material.

In general, the observed biofilms (Figure 6.8) presented very heterogeneous structures that covered practically 100 % of the surface area. There are some differences that were observed in the biofilms grown on the different materials. The biofilm on PVC had the highest amounts of EPS with holes due to shrinkage, or looked like a smooth coating that cracked probably during the dehydration process (Figure 6.8), and just a few bacteria were visualized on the top layers. EDS microanalysis confirmed the presence of biofilm on the surface (higher content of carbon) since C/Cl proportion increased (> 13.9) for the biofilm areas A1 and A2 than that of PVC material (1.4) (Table 6.12). For the other materials (SS, SiO_x, Excalibur and DLC), it was observed that surfaces were covered either with a thin organic layer with or without dispersed bacteria on top, or with thick cells agglomerates partially embedded in EPS. In addition, DLC was the only material where bacteria were forming filamentous colonies.

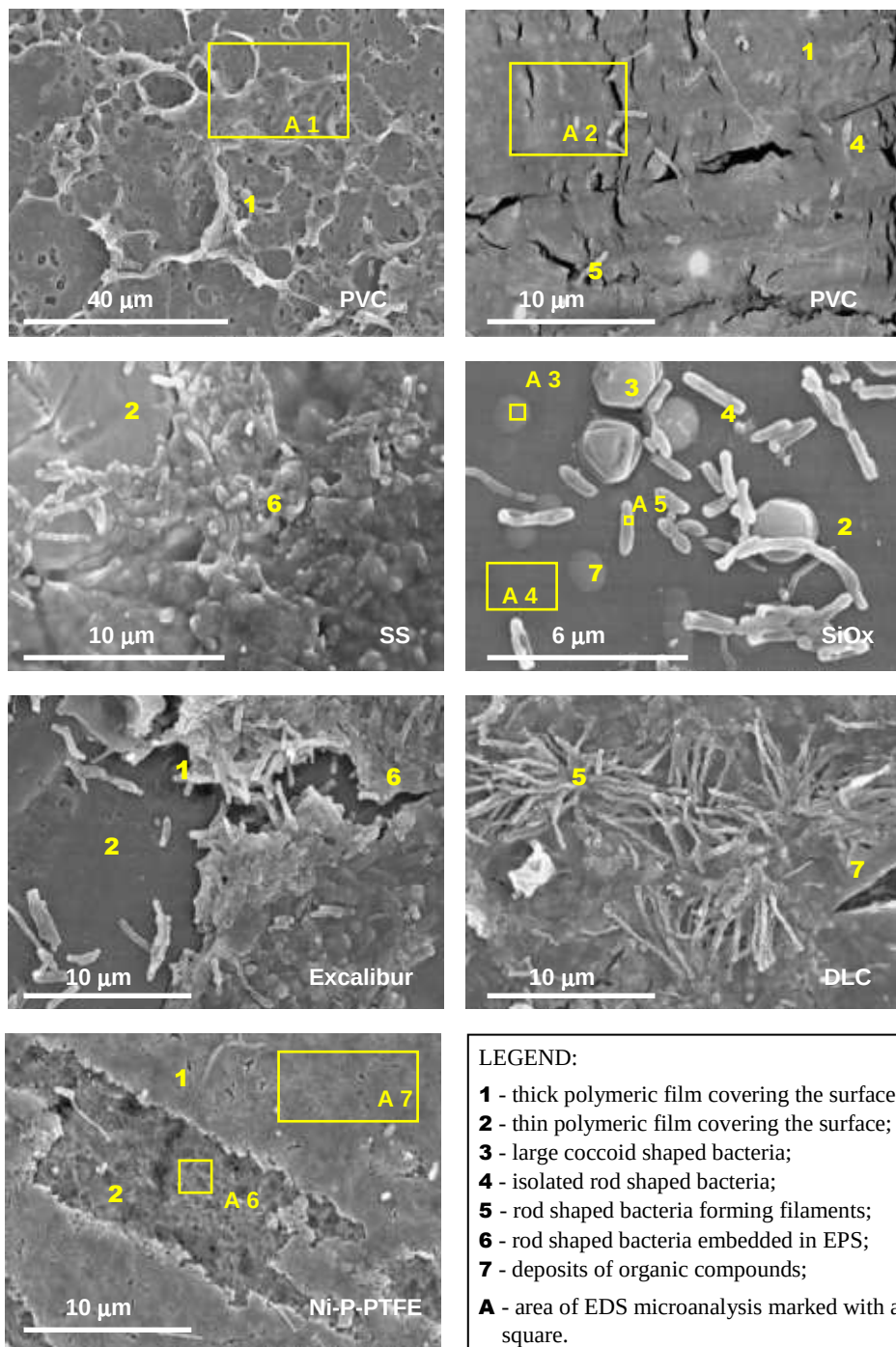


Figure 6.8 - SEM micrographics of biofilms grown on different surfaces collected at 1500 - 10000 magnification, with a working distance of 15 mm and an x-ray energy of 10-15 kV. EDS microanalysis was preformed in the areas marked with square.

Generally, all EDS results of biofilms indicated an increase of carbon content (Table 6.12 and Table 6.13) when compared to the results of the material surfaces (Table 6.6 and Table 6.7). It can be said that the lower the amount of elements composing the adhesion material, the higher the biofilm accumulation.

In some materials (SS, Excalibur and Ni-P-PTFE), thin organic coatings were observed, and thus, the material surface was still visualized (Table 6.13), raising the possibility of this being an artifact (biofilm removal) formed during biofilm preparation for SEM. The composition obtained for the areas A6 and A7 of the biofilm grown on Ni-P-PTFE support the observation that biofilm in area A6 was very thin.

Table 6.12 - Superficial composition of elements composing the PVC material obtained by EDS microanalysis.

Element	Elemental composition (%wt.)	
	Area A1 (PVC)	Area A2 (PVC)
C	87.0 ± 3.1	92.9 ± 2.8
S	9.1 ± 0.6	n.d.
Cl	2.3 ± 0.2	6.7 ± 0.3
Ca	1.6 ± 0.2	0.4 ± 0.2

n.d. - not detected.

Table 6.13 - Superficial composition of elements composing the stainless steel and the surface modified materials obtained by EDS microanalysis.

Element	Elemental composition (%wt.)				
	Area A3 (SiOx)	Area A4 (SiOx)	Area A5 (SiOx)	Area A6 (Ni-P-PTFE)	Area A7 (Ni-P-PTFE)
C	18.6 ± 6.1	6.8 ± 7.4	41.2 ± 4.0	15.3 ± 1.0	14.2 ± 1.0
F	n.d.	n.d.	n.d.	28.4 ± 1.3	14.7 ± 0.7
Si	81.4 ± 7.9	93.2 ± 9.2	58.8 ± 4.9	n.d.	n.d.
P	n.d.	n.d.	n.d.	6.0 ± 0.4	6.9 ± 0.4
Ni	n.d.	n.d.	n.d.	50.3 ± 2.0	64.1 ± 1.6

n.d. - not detected.

Large-cocoid and rod shaped bacteria were observed with very different spatial organizations: cocoid bacteria were generally dispersed on the top of biofilms formed on SiOx, DLC and SS; rod bacteria (present in all biofilms) were observed

either embedded in compact EPS, or forming colonies (with spiral-shape or not) adhered with EPS or were spread on the top of biofilm.

For the biofilm grown on SiOx surface, EDS was performed in three different areas which were: A3 corresponded to a material that looked different from bacteria, A4 in the organic smooth layer and A5 in a bacterium. The results proved that area A4 was the organic coating, A5 was a biological material with higher carbon content and A3 was also an organic compound composed only of carbon.

SEM preparation may create some artifacts on biofilm samples by drying completely the structures, such as, biofilm detachment, holes in the biofilm and EPS shrinkage (Fratesi *et al.*, 2004). These authors viewed also an untreated wet biofilm that showed an intact, hydrated, smooth matrix of EPS embedding cells. No holes were visible and no shrinkage artifacts were evident. Filaments, along with masses of microspheres and the smooth mucilaginous outer layer, also occur in wet samples studied by ESEM, and are, therefore, not dehydration artifacts.

In conclusion it seemed that the physicochemistry of materials surface affected biofilm structures although these relations were not that obvious. For instance, biofilm on PVC had apparently much more EPS than those formed on the other metallic based materials. It is also interesting to observe that the biofilm that accumulated less total cells/cm² presented a structure formed with filamentous colonies.

6.3.9 Microbial Composition

6.3.9.1 FISH

Samples of biofilm grown on different materials (PVC and stainless steel with and without surface modification by SiOx, Excalibur, DLC and Ni-P-PTFE) and of the reactor water were collected and analyzed through FISH (**Exp. 6.3**). The microbial diversity was obtained for the probed phylogenetic groups (Table 2.6) and the results were expressed as a percentage related to total cells stained with DAPI (Table 6.14).

In biofilms and reactor water, the bacteria belonging to the phyla of *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* totalized almost all DAPI-stained bacteria (80 - 90 %). *Beta*- and *Gammaproteobacteria* were the most predominant phylogenetic groups representing 37 to 53 % and 21 to 29 %, respectively. The exception was for the biofilm grown on Excalibur (where *Bacteroidetes* replaced

Gammaproteobacteria in the dominant groups with 33.8 %). Kalmbach *et al.* (1997b) and Schwartz *et al.* (2003a) observed also differences in bacterial populations grown on glass, PVC, HDPE and steel, although the dominant group was always the *Betaproteobacteria*. Nitrite oxidizing bacteria (NOB) and ammonia oxidizing bacteria (AOB) were also investigated and less than 7.7 % was present, being AOB fraction always higher than that of NOB.

The effect of hydrophobicity and roughness did not seem obvious, although it was expected that their effect was reduced for biofilms with longer lifetime and layers formed far from the materials surface.

Table 6.14 - Composition of the biofilm and water samples collected in **Exp. 6.3** in terms of each targeted phylogenetic group.

Phylogenetic groups		Fractions of phylogenetic groups (%)			
		PVC	SS	SiOx	Excalibur
<i>Bacteria</i> domain subgroups	<i>Alphaproteobacteria</i>	11.3 ± 3.1	1.7 ± 0.5	1.4 ± 1.0	1.6 ± 0.5
	<i>Betaproteobacteria</i>	48.2 ± 6.5	49.6 ± 6.7	45.3 ± 3.7	53.3 ± 6.3
	<i>Gammaproteobacteria</i>	25.6 ± 3.2	27.3 ± 2.5	21.9 ± 5.1	0.7 ± 0.1
	<i>Bacteroidetes</i>	2.3 ± 0.8	5.2 ± 1.0	7.5 ± 1.7	33.8 ± 2.7
	<i>Actinobacteria</i>	1.5 ± 0.6	2.2 ± 1.2	1.0 ± 0.2	1.2 ± 0.5
	AOB (<i>Nitrosomonadales</i>)	2.7 ± 1.1	3.1 ± 0.7	5.4 ± 1.3	2.7 ± 0.8
	NOB (<i>Nitrospirales</i>)	0.6 ± 0.2	1.2 ± 0.3	2.3 ± 0.9	0.7 ± 0.1

AOB - ammonia oxidizing bacteria belonging to *Betaproteobacteria*; NOB - nitrite oxidizing bacteria.

Table 6.14 (cont.) - Composition of the biofilm and water samples collected in **Exp. 6.3** in terms of each targeted phylogenetic group.

Phylogenetic groups		Fractions of phylogenetic groups (%)		
		DLC	Ni-P-PTFE	Reactor water
<i>Bacteria</i> domain subgroups	<i>Alphaproteobacteria</i>	3.5 ± 2.0	10.7 ± 2.0	12.5 ± 4.1
	<i>Betaproteobacteria</i>	45.4 ± 4.1	37.3 ± 5.6	42.1 ± 2.3
	<i>Gammaproteobacteria</i>	26.5 ± 2.6	29.3 ± 2.3	24.7 ± 1.9
	<i>Bacteroidetes</i>	5.3 ± 1.3	1.0 ± 0.4	3.0 ± 0.7
	<i>Actinobacteria</i>	2.0 ± 0.8	4.3 ± 2.0	3.5 ± 1.2
	AOB (<i>Nitrosomonadales</i>)	n.d.	n.d.	2.2 ± 1.0
	NOB (<i>Nitrospirales</i>)	2.6 ± 0.9	n.d.	1.7 ± 0.4

AOB - ammonia oxidizing bacteria belonging to *Betaproteobacteria*; NOB - nitrite oxidizing bacteria.
n.d. - not detected

6.3.9.2 DGGE

The same samples of biofilm grown on different materials (PVC and stainless steel with and without surface modification by SiOx, Excalibur, DLC, Ni-P-PTFE) and of the reactor water were analyzed for the total bacterial diversity using DGGE (Exp. 6.3). The DGGE gel of biofilm and reactor water samples is presented in Figure 6.9. Although with some exceptions, as discussed previously in chapter 3, each band on a precise gel position can be related to a different microorganism. The band patterns in each lane revealed a diverse microbial population.

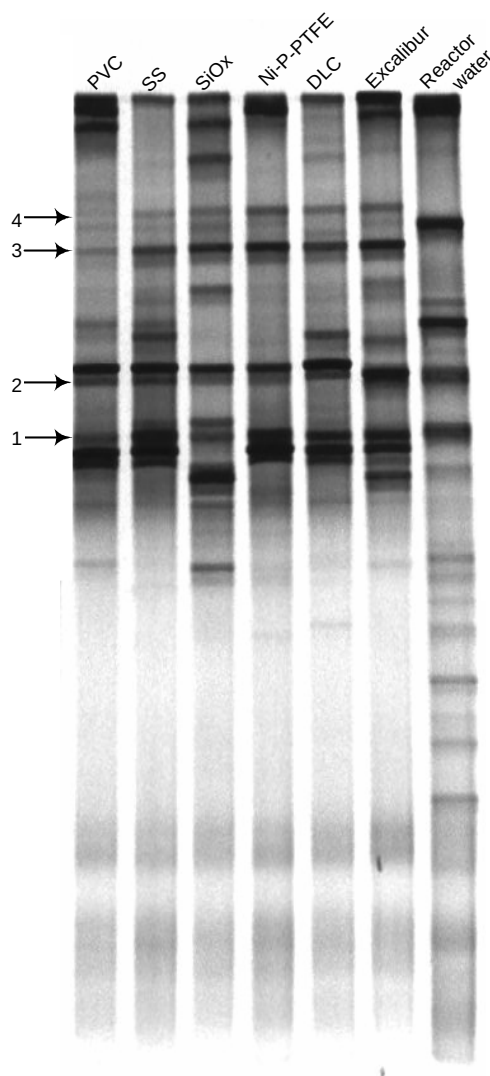


Figure 6.9 - DGGE patterns from 16S rDNA 180 bp fragments of biofilms grown on the different material surfaces and of reactor water collected in Exp. 6.3 after 3 months of operation.

The microbial population in the reactor water looked much more different and diverse than the biofilms. Its pattern revealed at least 17 different microorganisms, of which 7 were not present in any of the biofilms. Possibly, not all bacteria were able to be well incorporated in the biofilm due to their slow growth, reduced or inactive metabolic state, no cultivability capacity, cell damage, cell death, *etc...* Observing all the DGGE patterns, two microorganisms were common in all biofilm samples and in the reactor water (bands in positions 1 and 2) and two other (positions 3 and 4) were present only in the biofilms. The fact that the bands 3 and 4 were absent in the reactor water indicated that the corresponding microorganisms were not present or were in such a low amount that the extracted and amplified DNA fragment were not enough to be visualized in the gel.

Converting the band patterns to a binary matrix of presence/absence (1/0), a dendrogram (Figure 6.10) was constructed by cluster analysis as described in section 2.7. Among the biofilm samples, three clusters were obtained. The DGGE profiles of biofilms grown on Excalibur and SiOx, presented a similarity of 69 %, whereas those grown on PVC and SS showed 82 % of similarity. The highest percentage of similarity (89 %) was observed for the biofilm grown on Ni-P-PTFE and DLC. Also, the lowest similarity among the biofilms clusters is 52 %, which indicates that many bacteria were common in all communities.

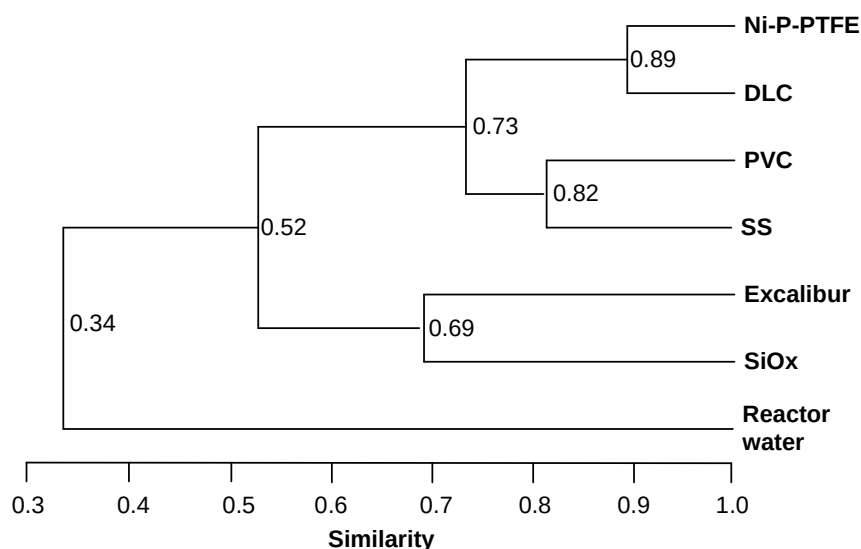


Figure 6.10 - Dendrogram representing the genetic similarity of the microbial community profile obtained by PCR-DGGE from the biofilm samples on the different material surfaces.

In accordance with the conclusions drawn from the DGGE patterns, reactor water was the most dissimilar sample (with only 34 % of similarity with cluster containing all the biofilm profiles). Comparing these clusters with the roughness parameters and with the surface energies, no evident correlations were obtained. The clusters combined either two materials that were hydrophilic and hydrophobic or two hydrophobic materials with different degrees of hydrophobicity. These associations seem to suggest that the thermodynamic approach based on the hydrophobicity cannot fully explain the bacterial adhesion, as already observed by other authors (Pereira *et al.*, 2000; Teixeira and Oliveira, 2000; Simões *et al.*, 2007). Several authors (Kalmbach *et al.*, 1997b, 1997a; Schwartz *et al.*, 1998) observed also that the microbial population in biofilm were affected by the surface materials although these effect was reduced with the biofilm lifetime.

6.3.10 Effect of Surface Material on Growth Kinetics

In order to assess the kinetics of biofilm development on different materials, tests were carried out in the Flow Cell (**Exp. 6.4**) where a higher number of biofilm and water samples were collected during the initial phase (transient period) of biofilm formation with HDPE and PVC as adhesion surfaces. The experimental data and the kinetic model are presented in Figure 6.11 for the biofilm grown on HDPE (**Exp. 6.4**). The results obtained for PVC were already presented in section 4.3.5.

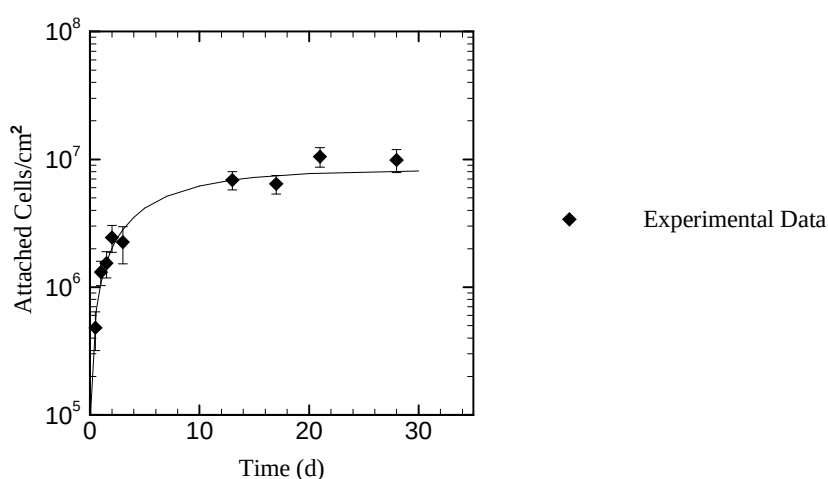


Figure 6.11 - Biofilm cells per unit surface area of biofilm grown under $u = 0.34$ m/s and $HRT = 12.0$ h on HDPE, in **Exp. 6.4**. Eq. (2.21) is represented by the continuous line.

The values of the specific growth rates obtained for biofilms grown on HPDE (Table 6.15) and on PVC (Table 4.3) were quite similar ($0.14 - 0.15 \text{ day}^{-1}$), meaning these surface materials had reduced effect on the kinetic parameters.

Table 6.15 - Specific cell growth rates at steady state for the biofilms grown on HDPE (Exp. 6.4) and on PVC (Exp. 4.2) under turbulent flow in Flow Cell reactor.

Kinetic parameters	HDPE	PVC
$(Xb)_{\max}$ (cell/cm ²)	$8.2 (\pm 1.3) \times 10^6$	$6.1 (\pm 2.5) \times 10^6$
Φ_p (cell/day)	$3.1 (\pm 0.9) \times 10^2$	$2.5 (\pm 1.4) \times 10^2$
μ^*_{biofilm} (day ⁻¹)	0.14 ± 0.05	0.15 ± 0.09
μ^*_{bulk} (day ⁻¹)	1.8 ± 0.1	1.8 ± 0.1
μ^*_{overall} (day ⁻¹)	1.3 ± 0.2	1.4 ± 0.2

6.4 Conclusions

a) Surface energies and hydrophobicity parameters were determined after contact angle measurements. Independently of the criteria based on the value of the contact angle with water, on the surface energy components or on the variation of free energy of hydrophobicity, the conclusions were similar. Stainless steel was hydrophilic, PVC, PEX, SiOx and DLC were low hydrophobic surfaces and HDPE, PP, Excalibur and Ni-P-PTFE were high hydrophobic surfaces. The mean roughnesses varied from 0.07 to 1.59 μm and increased in the following order $\text{DLC} = \text{SiOx} < \text{SS} = \text{Ni-P-PTFE} = \text{PVC} < \text{PEX} = \text{PP} = \text{Excalibur} < \text{HDPE}$.

b) EDIC/EF Microscopy was a useful and fast technique to observe surfaces without the need of prior surface preparation. However SEM/EDS presented some important advantages (higher magnifications and surface composition) that allowed a better knowledge of the observed structures. All surface coatings were homogeneous and covered the metallic matrix with thickness $\geq 1 \mu\text{m}$, except DLC and SiOx that were thinner or presented variable thickness along the surface.

c) Studies conducted in stagnant and flow systems showed that steady state biofilms, grown on PVC, PEX, HDPE and PP, presented higher ($10 \times$) total cells per unit surface area in the flow reactor than in stagnant system. Independently of the system and of the material, the fraction of metabolically active cells (18.9 - 32.8 %) was higher than the cultivable fraction in the biofilm (10.6 - 16.6 %).

d) In the 2 months-old biofilms formed in the Batch reactor, the effect of roughness and of hydrophobicity was negligible. However in the flow system, total cells per

unit surface area increased with the roughness and with the hydrophobic character among the polymeric materials. Principal component analysis also indicated that these parameters were affecting biofilm accumulation differently, depending on the hydrodynamic regime. In flow systems, the most similar materials were PVC and PEX (with closer values of roughness and hydrophobicity than those of HDPE and PP). The material with the highest roughness (HDPE) presented the highest biofilm accumulation potential. Comparing the materials with roughnesses around 0.15-0.80 μm , the most hydrophobic surface (PP) accumulated more biofilm cells. However, these two parameters were not so important in stagnant regime.

e) For the biofilms grown on stainless steel, with and without surface modification (SiOx, Excalibur, DLC and Ni-P-PTFE), the effect of roughness and of hydrophobicity on biofilm accumulation was not clear, probably due to large differences in their chemical composition.

f) SEM analysis and surface composition showed high contents in carbon and very different structures in biofilms covering all the material surfaces, basically composed of bacteria and polymeric substances. The effect of roughness and hydrophobic character on the 3-months old biofilm structures was not obvious.

g) *Beta* and *Gammaproteobacteria* were the most predominant phylogenetic groups present in biofilms and in reactor water, excepting the biofilm community on Excalibur that was composed mainly by *Betaproteobacteria* and *Bacteroidetes*. DGGE patterns showed a higher diversity for the planktonic bacteria when compared to those attached to the different surfaces. At least half of the types of bacteria were present in the biofilm communities attached to the different materials. No significant correlations were obtained between the microbial populations and the surface properties (hydrophobicity and roughness).

h) The specific cell growth rate in the biofilm, μ^*_{biofilm} , was determined for biofilms growing on HDPE and PVC and the values were very similar (0.14 - 0.15 day^{-1}). This suggests that the material effect on biofilm growth in steady state was negligible. The specific cell growth rate in the water was 1.8 day^{-1} , independently from the surface material. This value was similar to the inverse of the hydraulic residence time, which is according with the chemostat principles.

These conclusions can be of importance in maintaining a good quality of drinking water. Independently from the material and from the hydrodynamic regime (static versus flow), the biofilm will grow and accumulate attached to pipe surfaces.

Although bacterial adhesion did not correlate perfectly with any studied parameter, it can be stated that surfaces that are rough, or chemically instable in water or with higher hydrophobicity should be avoided since they have a higher biofilm accumulation potential.

7. Conclusions and Perspectives

7.1 Conclusions

Water-related companies must survey the characteristics of drinking water through the whole networks until consumer's tap in order to guarantee a good physicochemical and microbiological quality. Drinking water biofilms are surely a major problem since they promote biomass accumulation, shield microorganisms, decrease the disinfectants efficacy and promote biomass accumulation. In addition, pathogens may incorporate and accumulate within biofilms and the attached biomass may be released to the water phase. Therefore, the water quality will be lowered while the probability of occurrence of health risky events will rise. These facts evidence the interest on acquiring a good knowledge of biofilms behavior under the same conditions as those observed in drinking water systems.

In this work, attached and suspended bacteria accumulation was followed in reactors fed with drinking water coming from the pipe in terms of their physicochemical and microbiological parameters. This inlet water was also analyzed revealing that was according the Portuguese law. The seasonal time and stagnancy affected the drinking water characteristics, *i. e.*, high temperatures increased the organic carbon content, the total cell density and the percentages of cells with metabolic activity and cultivability capacity. Although it was suspected that *Pseudomonas aeruginosa* contaminated the reactors fed with drinking water, no

further detection was confirmed in several drinking water samples indicating probably a very low level of contamination. Fluorescent *in situ* hybridization and phenotypic characterization were in accordance that microbial populations were mainly composed of *Proteobacteria*, although *Bacteroidetes* and *Actinobacteria* were also present. Denaturing Gradient Gel Electrophoresis showed also the great complexity of the water microbial populations.

Biofilm accumulation and suspended bacteria were followed under steady state hydraulic conditions (turbulent and laminar flow and stagnant conditions) in reactors fed with drinking water. Flow reactors with different designs (Flow Cell and Propella) were used and had similar performance as regards bacterial accumulation on surfaces, while in stagnant conditions, bacteria accumulation was much lower. Increasing the water velocity enhanced detachment events, and consequently increased bacterial density and decreased steady state values of wall cell accumulation. Also, biofilm cell growth rates were lower for higher velocities. The effect of residence time on biofilm was significant and similar to the effect of stagnancy in drinking water. These results suggest possible metabolic changes related to differences in shear stress over biofilms.

Biofilm accumulation and suspended bacteria were followed under unsteady state hydraulic conditions (alternating between flow and no-flow conditions). Stagnancy promoted biomass accumulation, in suspension and in biofilms, and caused a higher increase in the cultivability of cells than in their metabolic activity. Biofilms formed under turbulent or laminar flow behaved differently due to differences in structure and cohesion. Therefore, both biofilms accumulated cells during stagnancy periods that release to the water after restarting the flow. Under alternating stop/flow conditions biofilm reached similar numbers of those formed under steady state flow conditions operational. The percentages of metabolically active and of cultivable cells were observed to decrease with the biofilm depth and lifelength. Biofilm microbial populations were very similar to those found in drinking water and *Betaproteobacteria* was the most predominant group, independently from the hydraulic regime (turbulent or laminar; stagnancy or flow conditions). The succession of microbial population over time was highly affected by hydraulic conditions since it influences the structure of biofilms.

The effect of materials on biofilm growth was studied for several polymeric (PVC, PEX, SiOx and DLC) and for stainless steel with and without surface modification (SiOx, Excalibur, DLC and Ni-P-PTFE coatings). The hydrophobic character and the roughness affected biofilm accumulation on polymeric materials in flow

systems and were negligible in stagnant conditions. For the biofilms grown on the metallic based materials, the effect of roughness and of hydrophobicity on biofilm accumulation was not clear, probably due to shorter range of roughness and high differences in material composition. SEM/EDS analysis of biofilms revealed high contents in carbon and very different structures covering all the material surfaces. Microbial composition of biofilms was affected by the surface materials, although the dominant phylogenetic group was *Betaproteobacteria*. DGGE patterns revealed a higher diversity for the planktonic bacteria when compared to those attached.

These conclusions can be of help in keeping a good quality of drinking water systems, mainly in cases where there are significant periods of stagnancy, such as in seasonal hotels, some hospital showers and water reservoirs in buildings. High temperatures and variations in water velocity should be avoided or, at least, the first water coming from the tap not collected. Although biofilm accumulation did not correlate perfectly with material properties, it can be stated that surfaces that are rough, or chemically instable in water or high hydrophobic should be avoid.

7.2 Perspectives

Based on the experimental work and the results obtained in this thesis, there are several perspectives and recommendations for future work to be carried out in three major subjects. The first will be on the identification of microbial populations of the biofilm from the beginning until it reaches the steady state. The second subject will be on the study of mechanisms and molecules involved on the adhesion of the bacteria identified in the first subject. Finally, the third should be devoted the development of a material that truly minimizes the biofilm attachment. A more detailed explanation of these subjects and some recommendations about the tests and techniques are presented in the following sections. However, it should be emphasized that studies should be performed under conditions approaching those observed in real water systems, *i. e.* the studies should be performed with the heterogeneous bacterial communities coming from the tap water; the velocity, temperature and residence time should be similar to those expected in real water systems. Also, bacterial samples should be minimally handled in order to reduce the perturbations in the real microbial populations and avoid unbiased results. In addition, enough sample material should be collected to obtain results far from the detection limits of techniques.

Identification of microbial populations in biofilms.

The microbial populations may be identified using DGGE and FISH. These two techniques were not easy to implement because drinking water bacteria is generally present in very low numbers and with low contents of nucleic acids due to the fact that the environment is oligotrophic and disinfected. FISH analysis could be applied using the same probes used in this work although the results are global in terms of different phylogenetic groups. Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) could be tested in parallel in order to evaluate if the detection of cells with low ribosomal content are significantly enhanced (Wilhartitz *et al.*, 2007). For the DGGE technique, it is recommended to test more extraction techniques with higher DNA recovery efficiency. After running the amplified DNA samples on the DGGE gels, bands may be excised and the corresponding DNA sequences identified by gene sequencing. These sequences may be aligned and compared with those in nucleotide databases in order to identify the bacteria. An interesting possibility is to clone directly the amplified DNA products to construct clone libraries. Single strand conformation polymorphism can be used to screen the clone sequences to select those unique clones for further sequencing to achieve their taxonomic results.

Adhesion - mechanisms and molecules.

After identifying the most common species present in drinking water biofilms, it will be easier to study the mechanisms and the molecules that are involved in the adhesion to the pipe walls. This subject may be initiated by identifying the extracellular polysaccharides, proteins, lipids and sugars that surrounds the bacteria in suspension and adhered (Neu *et al.*, 2002; Georgiou *et al.*, 2008). Staining these molecules with specific lectins/dyes will permit their spatial observation using the new technologies in Microscopy (ESEM, AFM, ...).

Development of a new material with reduced biofilm growth potential.

Until now and to our knowledge, it seems that materials with low biofilm growth potential are those that do not “react” with drinking water or biofilm bacteria (e.g. PVC and stainless steel) and are very smooth. However, drinking water biofilm accumulates anyway which reveals that probably the pipe surface is not that important for the most inner biofilm layers (far away from the surface). Nowadays it is possible to build “responsive” surfaces, which adjust their physicochemical properties to variations in the environment (e.g. electric field, chemical composition, physical stimulus,...). These materials that have variations in their

physicochemical properties probably will not allow good adherence of bacteria to the surface. Also, superhydrophobic materials (with contact angles with water higher than 150°) could be tested.

This task is probably not economically feasible to incorporate public drinking water systems and networks, but probably useful in other applications that need water with high quality standards, such as in pharmaceutical and medical areas.

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Appendix - Complementary Data and Results

In Appendix are presented the procedures for the preparation and the composition of some solutions that were used during the presented experimental work, as well as some experimental data that may be useful to complement this thesis.

A.1 Culture Media

R₂A medium was prepared from a reactant mixture (Merck 1.00416) with the composition indicated in Table A.1. R₂B was prepared with the same composition of R₂A without agar. The media were sterilized in an autoclave at 121 °C and 1.2 bar.

Table A.1 - Composition of 1 x R₂A medium.

Components	Conc. (g/L)	Components	Conc. (g/L)
Yeast extract	0.5	Sodium pyruvate	0.3
Proteose-peptone	0.5	di-potassium hydrogen phosphate	0.3
Casein hydrolysate	0.5	Magnesium sulphate	0.05
Glucose	0.5	Agar	12.0
Starch soluble	0.5		
pH = 7.2			

A.2 Composition and Preparation of Solutions for FISH

The composition and the preparation of all solutions used in FISH procedure are indicated in the following table.

Table A.2 - Composition and preparation of solutions used in FISH procedure.

Solution	Preparation
5 M NaCl	All this solutions were prepared according the indicated composition, divided in small portions and sterilized by autoclaving at 121°C and 1.2 bar, except the SDS, pipemidic acid and lisozyme solutions that was sterilized by 0.2 µm membrane filtration.
1 M Tris/HCl pH 8.0	
10 % wt. SDS	
0.5 M EDTA pH 8.0	
TE (10 mM Tris-HCl; 1mM EDTA; pH = 8)	
10 mg/mL lisozyme (SIGMA,L6876) in TE buffer	
10 g/L of pipemidic acid (SIGMA P7903) in NaOH 0.05 M	
10 × R ₂ B (table A1)	
1× Phosphate buffer 200mM	Prepare 27.6 g/L of NaH ₂ PO ₄ solution. Prepare 35.6 g/L of Na ₂ HPO ₄ solution. Mix 20 mL of NaH ₂ PO ₄ with 80 mL of Na ₂ HPO ₄ . Adjust pH to 7.2 - 7.4.
3 × Phosphate buffer saline (PBS)	Prepare a solution with 390 mM NaCl and 30 mM phosphate buffer. Adjust pH to 7.2 - 7.4.
Paraformaldehyde fixative 4 % wt. in PBS	Add 0.4 g of paraformaldehyde (SIGMA P6148) to 6.5 mL of dH ₂ O. For a better dissolution add one drop of 2 M NaOH solution, heat at 60 °C and stir for 1-2 min. Add 3.3 ml of 3xPBS. Adjust pH=7.2 with HCl 2 M. Filtrate solution through a 0.2 µm membrane filter. Quickly cool down to 4 °C and store in the refrigerator in aliquots for use in the following month.

The buffers composition for the hybridization and washing steps are indicated in the following tables according the formamide stringencies of each FISH probe.

Table A.3 - Hybridization buffer composition according the formamide (FA) stringency of each applied probe.

Solutions	Volume (μL)				
	20 % FA	25 % FA	35 % FA	50 % FA	55 % FA
5 M NaCl	180	180	180	180	180
1 M Tris/HCl pH 8,0	20	20	20	20	20
10 % wt. SDS	2	2	2	2	2
Formamide	400	500	700	1000	1100
MQ water	1200	1100	900	600	500

Table A.4 - Washing buffer composition according the formamide (FA) stringency of each applied probe.

Solutions	Volume (μL)				
	20 % FA	25 % FA	35 % FA	50 % FA	55 % FA
5 M NaCl	2150	1490	700	180	100
1 M Tris/HCl pH 8,0	1000	1000	1000	1000	1000
10 % wt. SDS	50	50	50	50	50
0.5 M EDTA pH 8.0	500	500	500	500	500
MQ water	Add until 50 mL				

A.3 PCR Reaction Mixtures

The PCR mixtures were prepared simultaneously for all reactions and immediately before reacting under the thermocycler. All the reactants aliquots were placed in ice to avoid deterioration. The total mixture divided in volumes of 49 μL in each sterile 200 μL Eppendorf tube and then, 1 μL of the DNA template from the sample was added. The composition of each reaction mixture was the indicated in Table A.5.

Table A.5 - Composition of reaction mixture for the PCR.

Reactants	1 reaction
25 mM MgCl_2	6 μL
10x Taq buffer with KCl	2.5 μL
10x Taq buffer with $(\text{NH}_4)_2\text{SO}_4$	2.5 μL
DMSO	2.5 μL
10 mM dNTPs	1 μL
10 μM Primer F	1.5 μL
10 μM Primer R	1.5 μL
1U/ μL Taq polymerase	1 μL
DNA template	1 μL
UPF water	30.5 μL
Total volume	50 μL

For the nested PCR, the first and the second reaction mixtures in each Eppendorf tube are presented in Table A.6. For the 1st PCR cycle, the primers used were the 27f and the 1492r with a final volume of 25 μ L. In the 2nd cycle, the primers were the 338f and the 518r and the final volume was 50 μ L.

Table A.6 - Composition of reaction mixture for the two sequential cycles in the nested-PCR.

Reactants	1 st PCR cycle (1 reaction)	2 nd PCR cycle (1 reaction)
25 mM MgCl ₂	3 μ L	6 μ L
10x Taq buffer with KCl	1.25 μ L	2.5 μ L
10x Taq buffer with (NH ₄) ₂ SO ₄	1.25 μ L	2.5 μ L
DMSO	1.25 μ L	2.5 μ L
10 mM dNTP's	0.5 μ L	1 μ L
10 μ M Primer F	2.5 μ L	5 μ L
10 μ M Primer R	2.5 μ L	5 μ L
1U/ μ L Taq polymerase	1.25 μ L	2.5 μ L
DNA template*	2.5 μ L	3 μ L
UPF water	9 μ L	21 μ L
Total volume	25 μ L	50 μ L

* - DNA template in the 1st PCR is the extracted DNA from the samples. The template in the 2nd PCR is the product obtained in the 1st PCR.

A.4 Composition of DGGE Gel with Low and High Concentration

DGGE gel was prepared by mixing two separate gels with low and high concentration that will polymerize overnight after adding TEMED and ammonium persulfate at room temperature, whose composition was indicated in Table A.7.

Table A.7 - Composition of DGGE gels of low and high concentration.

Reactants	35 % gel (L)	60 % gel (H)
Urea (Biorad #161-0800)	2.352 g	4.032 g
50 x TAE (Biorad)		
1x = 40 mM Tris, 20 mM acetic acid, and 1 mM EDTA (pH 8.0)	320 µL	320 µL
Formamide (Sigma F7508)	2.24 mL	3.84 mL
40 % polyacrylamide (acrylamide:bisacrylamide 37.5:1)	3.20 mL	3.20 mL
Deionized water	Add until 16 mL	Add until 16 mL

Table A.8 - Volumes of the polymerization agents added to the gels of low and high concentration immediately before casting.

Polymerization agents	35 % gel (L)	60 % gel (H)
Tetramethylethylenediamine (TEMED, Biorad #161-0800)	14 µL	14 µL
10 % wt. ammonium persulfate (Biorad #161-0700)	140 µL	140 µL

A.5 Glutaraldehyde Solution for SEM Preparation

The glutaraldehyde solution was used for biofilm fixation prior SEM observation and was prepared at 3 % wt. in sodium cacodylate buffer pH = 7.2 as indicated in Table A.9.

Table A.9 - Preparation of glutaraldehyde fixative used to prepare biological samples for SEM visualization.

Solution	Preparation
Sodium cacodylate buffer pH = 7.2	- weight 1.07 g of sodium cacodylate trihydrate ($\text{Na}(\text{CH}_3)_2\text{AsO}_2 \cdot 3\text{H}_2\text{O}$, Merck 20840) and dilute in 50 mL of distilled water. - add 4.15 mL of HCL 0.1 N and dilute to 100 mL.
Glutaraldehyde fixative	dilute 12 mL of glutaraldehyde solution 25% (Merck 1.04239) to 100 mL of sodium cacodylate buffer pH = 7.2

A.6 EDIC and SEM Micrographics of Material Surfaces

In this section, complementary pictures of the clean surfaces obtained by EDIC-EF and SEM techniques are presented.

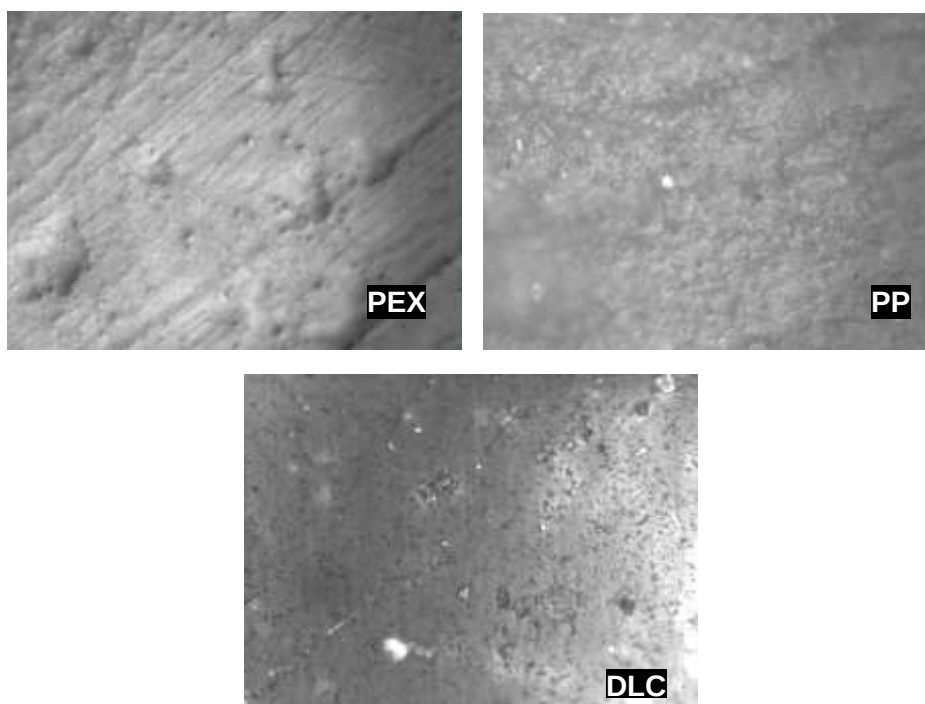


Figure A.1 - Materials surface observed under 1000 magnification with an EDIC-EF microscope: PVC, PEX, HDPE, PP, SS, SiO_x, AC Excalibur, DLC, Ni-P-PTFE (Area = 90.0 × 67.2 μm²).

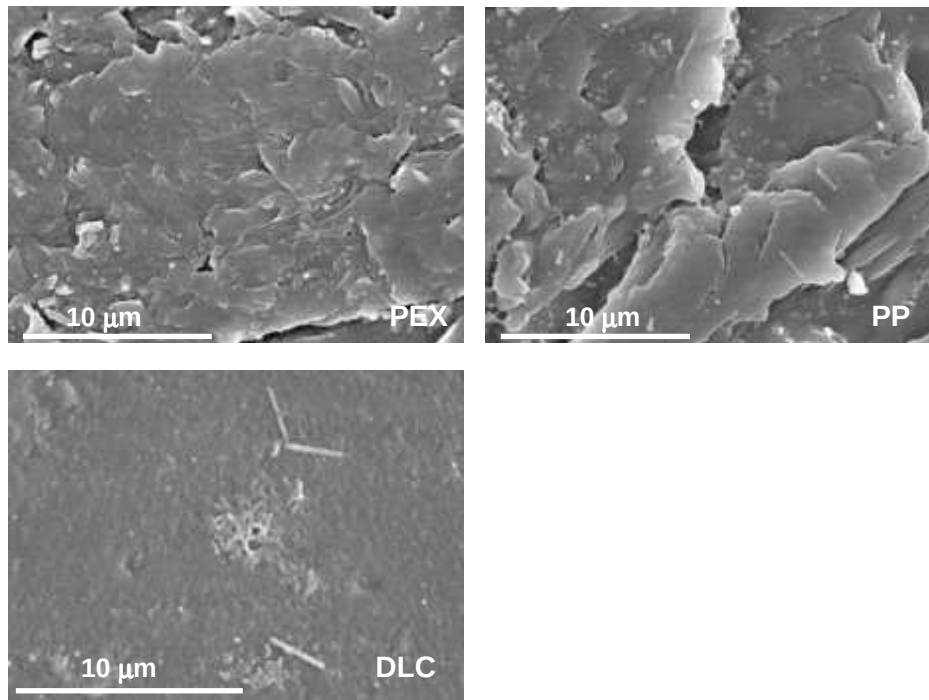


Figure A.2 - SEM micrographics of material surfaces at 5000 to 15000 magnification, with a working distance of 15 mm and an x-ray energy of 10-15 kV.

A.7 Biofilm Accumulation on Adhesion Materials

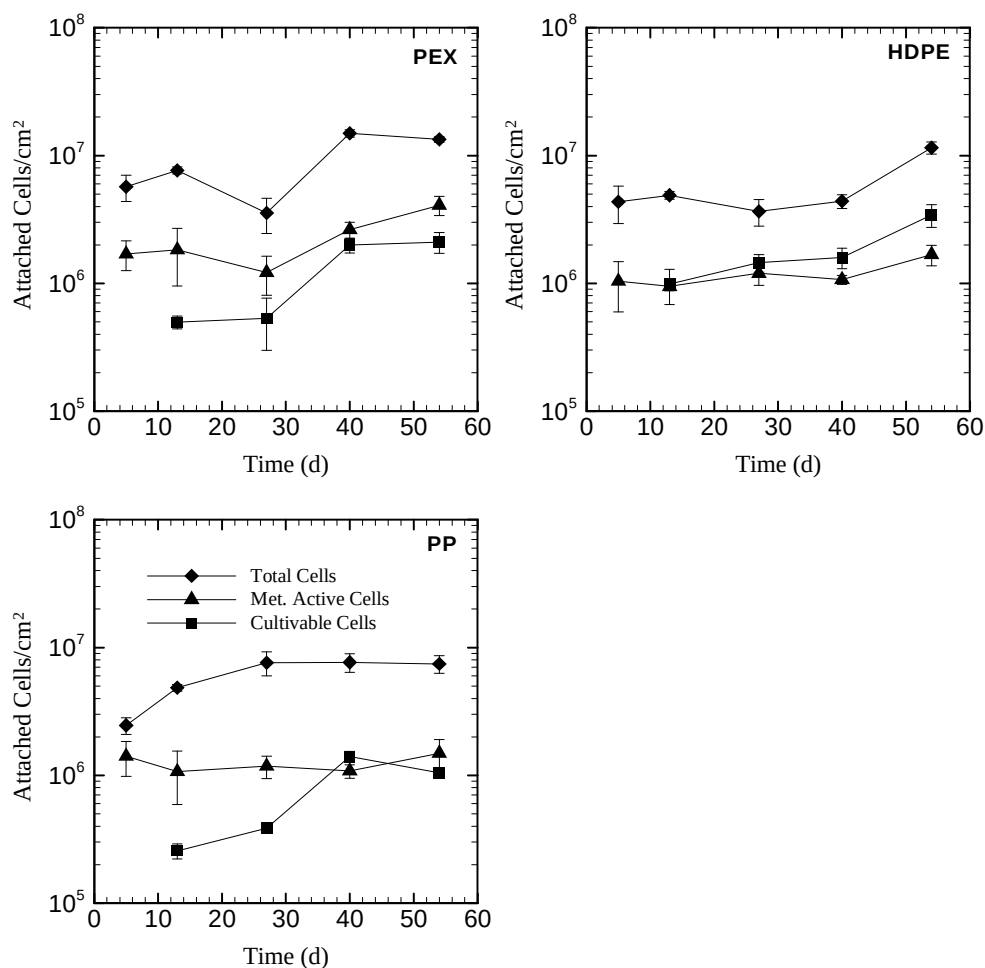


Figure A.3 - Attached cells per unit surface area (total, metabolically active and cultivable cells) for biofilms grown on the different polymeric materials - PEX, HDPE and PP - in the Batch reactor (**Exp. 6.1**).

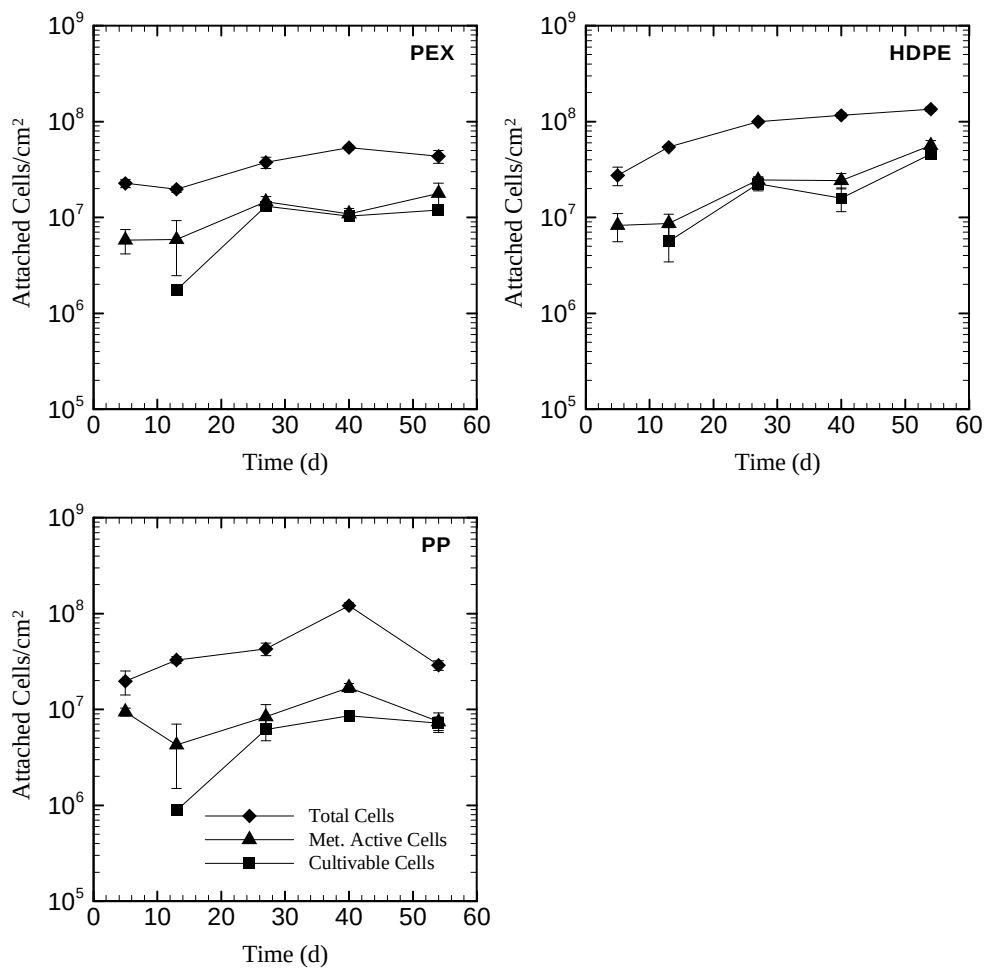


Figure A.4 - Attached cells per unit surface area (total, metabolically active and cultivable cells) for biofilms grown on different polymeric surfaces - PEX, HDPE and PP - in the Flow Cell reactor (Exp. 6.2).

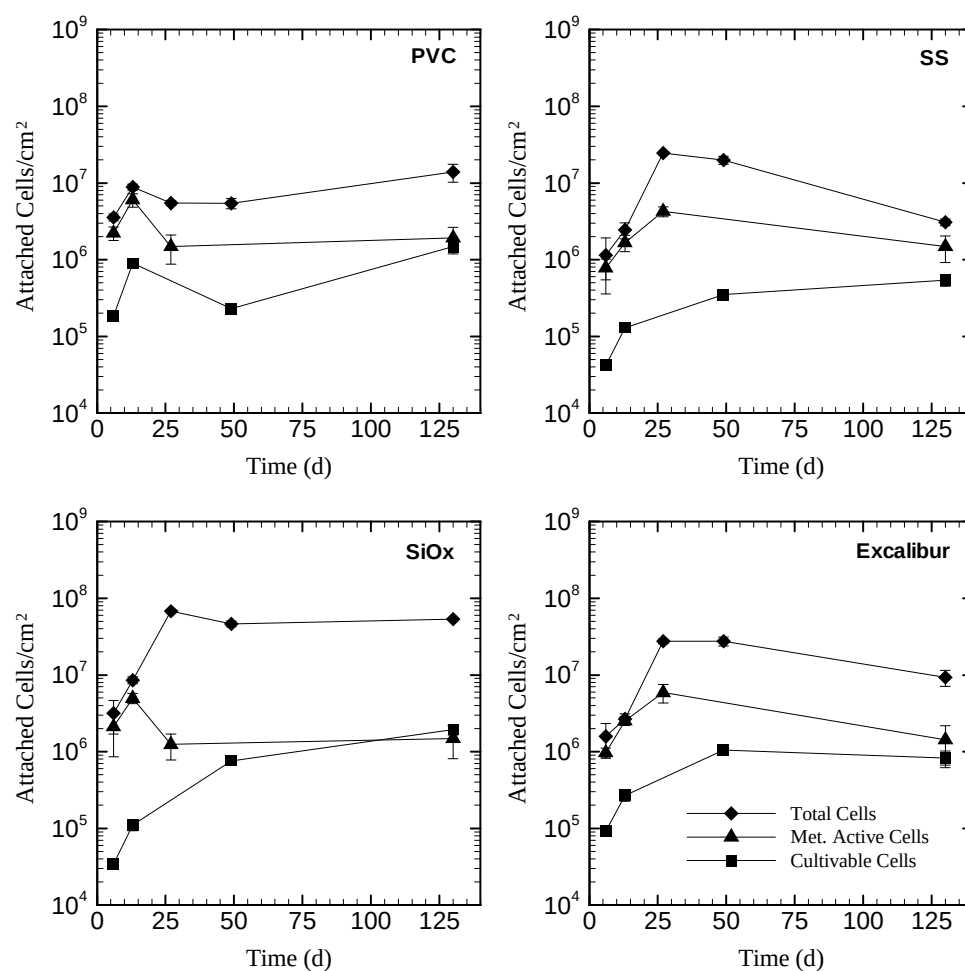


Figure A.5 - Attached cells per unit surface area (total, metabolically active and cultivable cells) for biofilms grown on PVC, SS and on different surface modified stainless steels - SiOx, Excalibur, DLC and Ni-P-PTFE coatings - in the Flow Cell reactor in **Exp. 6.3**.

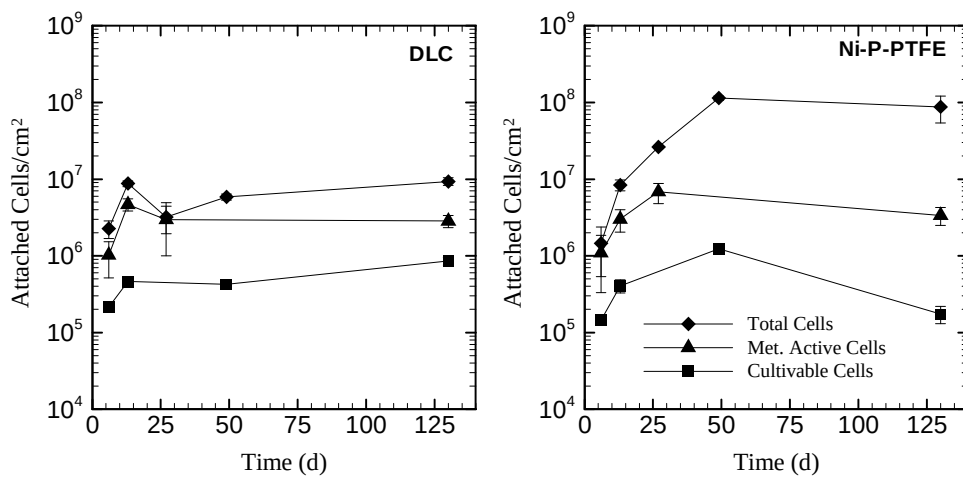


Figure A.5 (cont.) - Attached cells per unit surface area (total, metabolically active and cultivable cells) for biofilms grown on PVC, SS and on different surface modified stainless steels - SiOx and Excalibur, DLC and Ni-P-PTFE coatings - in the Flow Cell reactor in **Exp. 6.3**.

A.8 SEM Micrographics of Biofilms

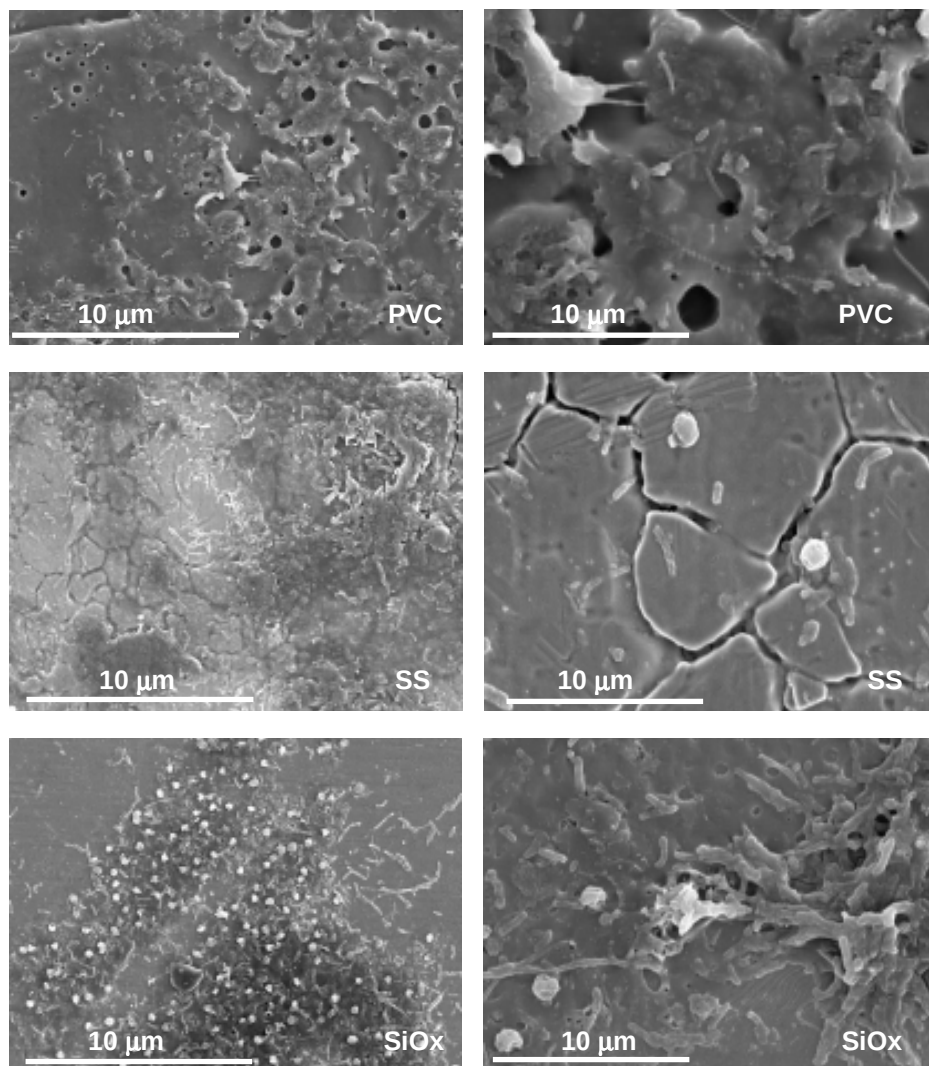


Figure A.6 - SEM micrographics of biofilms grown on several material surfaces at 5 000 - 10 000 magnification, with a working distance of 15 mm and an x-ray energy of 10-15 kV.

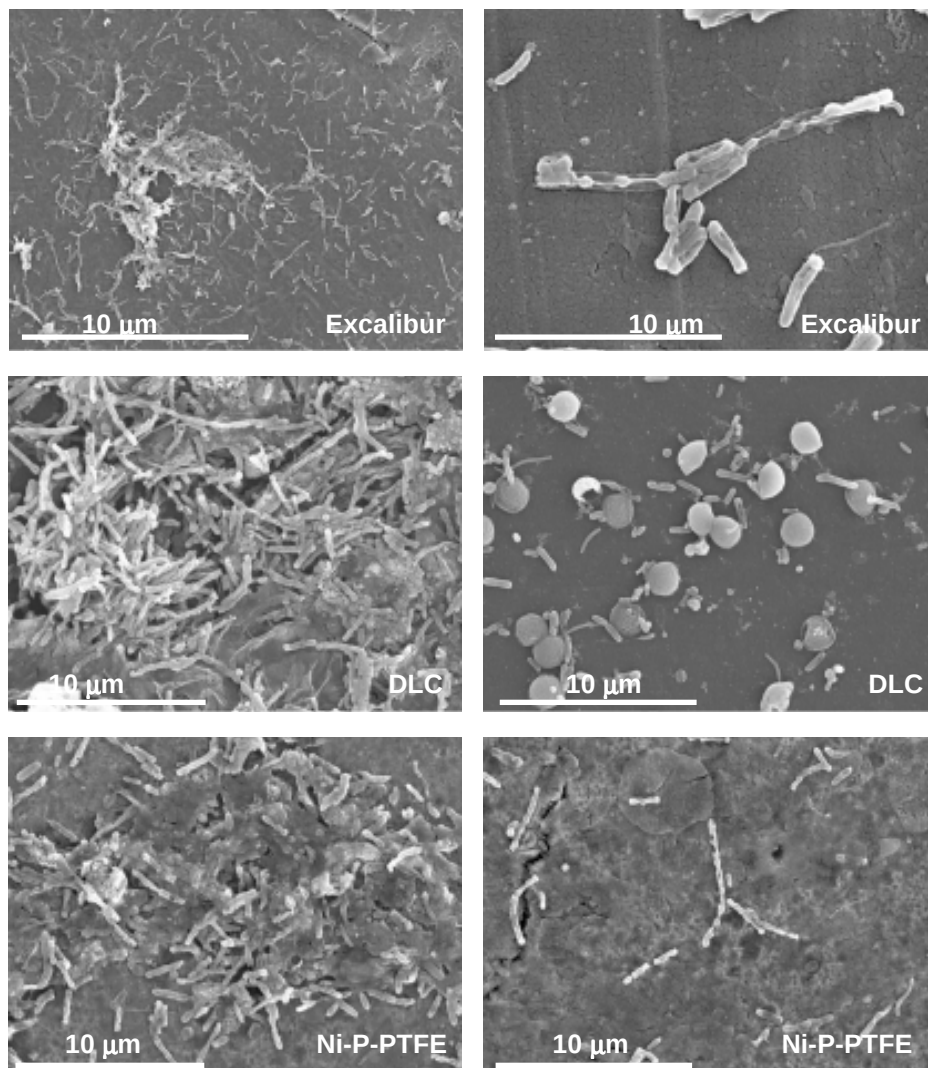


Figure A.6 (cont.) - SEM micrographics of biofilms grown on several material surfaces at 5 000 - 10 000 magnification, with a working distance of 15 mm and an x-ray energy of 10-15 kV.

A.9 EDS Spectra of Material Surfaces and Biofilms

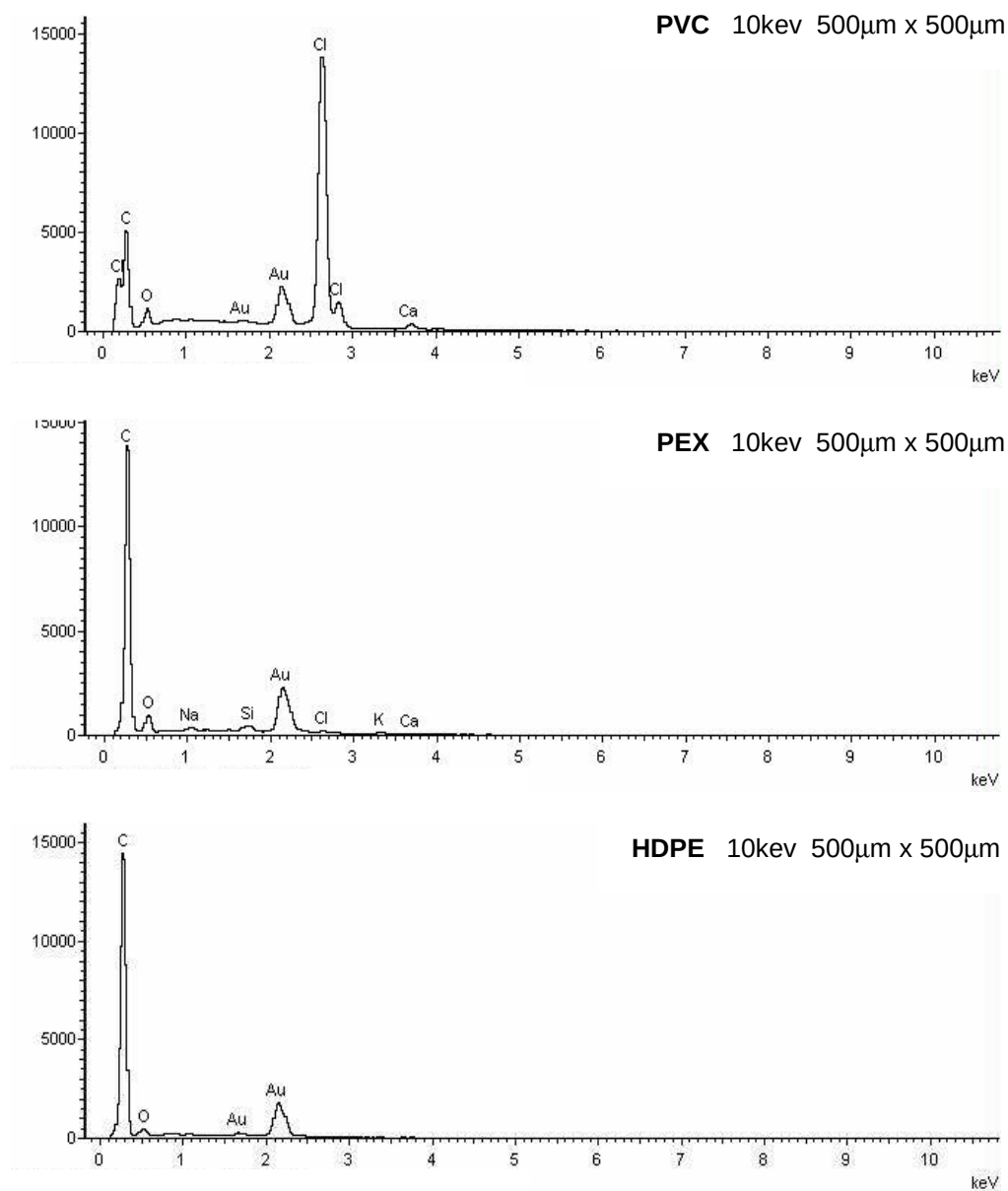


Figure A.7 - Spectra of elements obtained with EDS microanalysis by an X-ray energy of 10-15 keV for the different material surfaces.

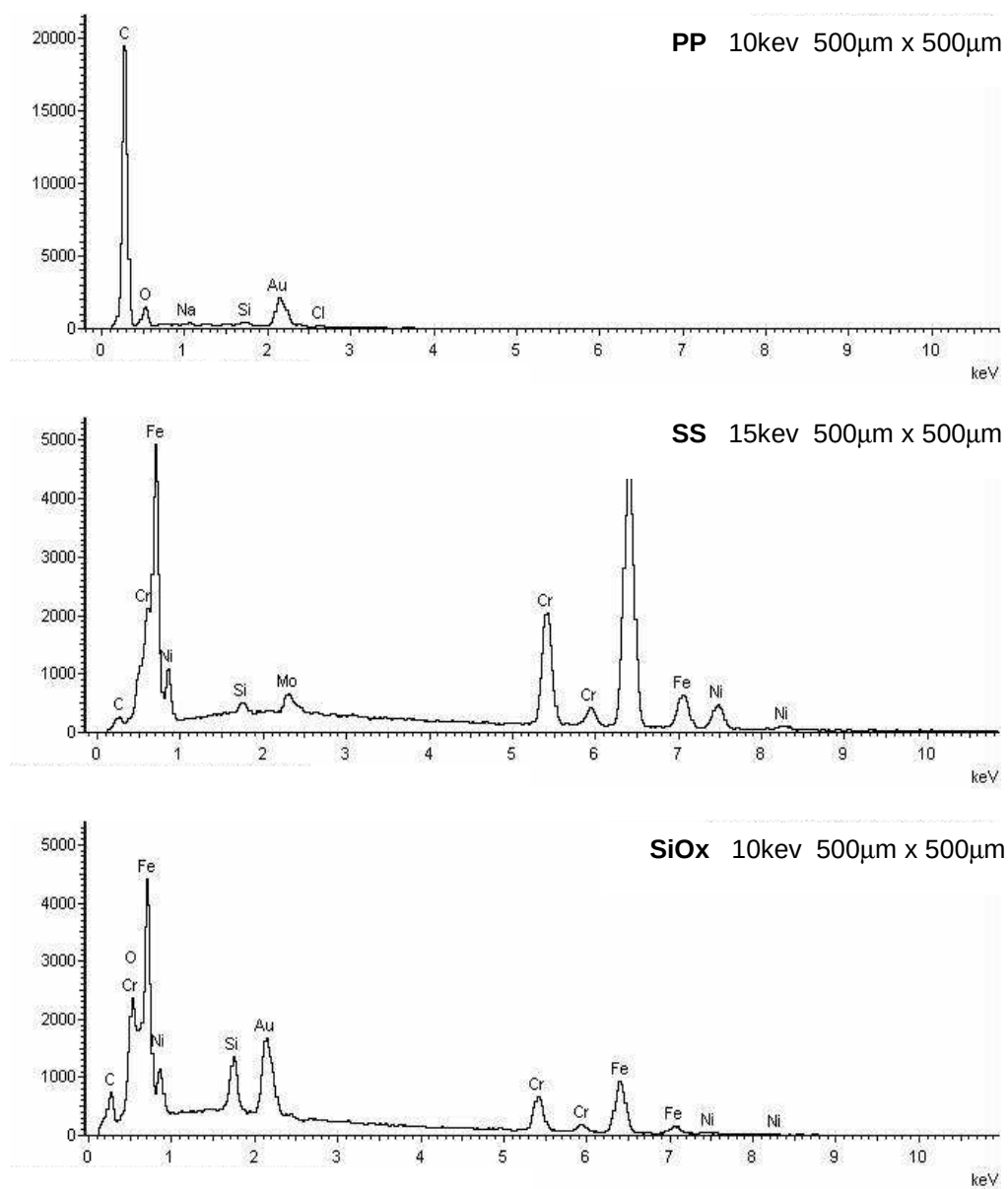


Figure A.7 (cont.) - Spectra of elements obtained by EDS microanalysis with an X-ray energy of 10-15 keV for the different material surfaces.

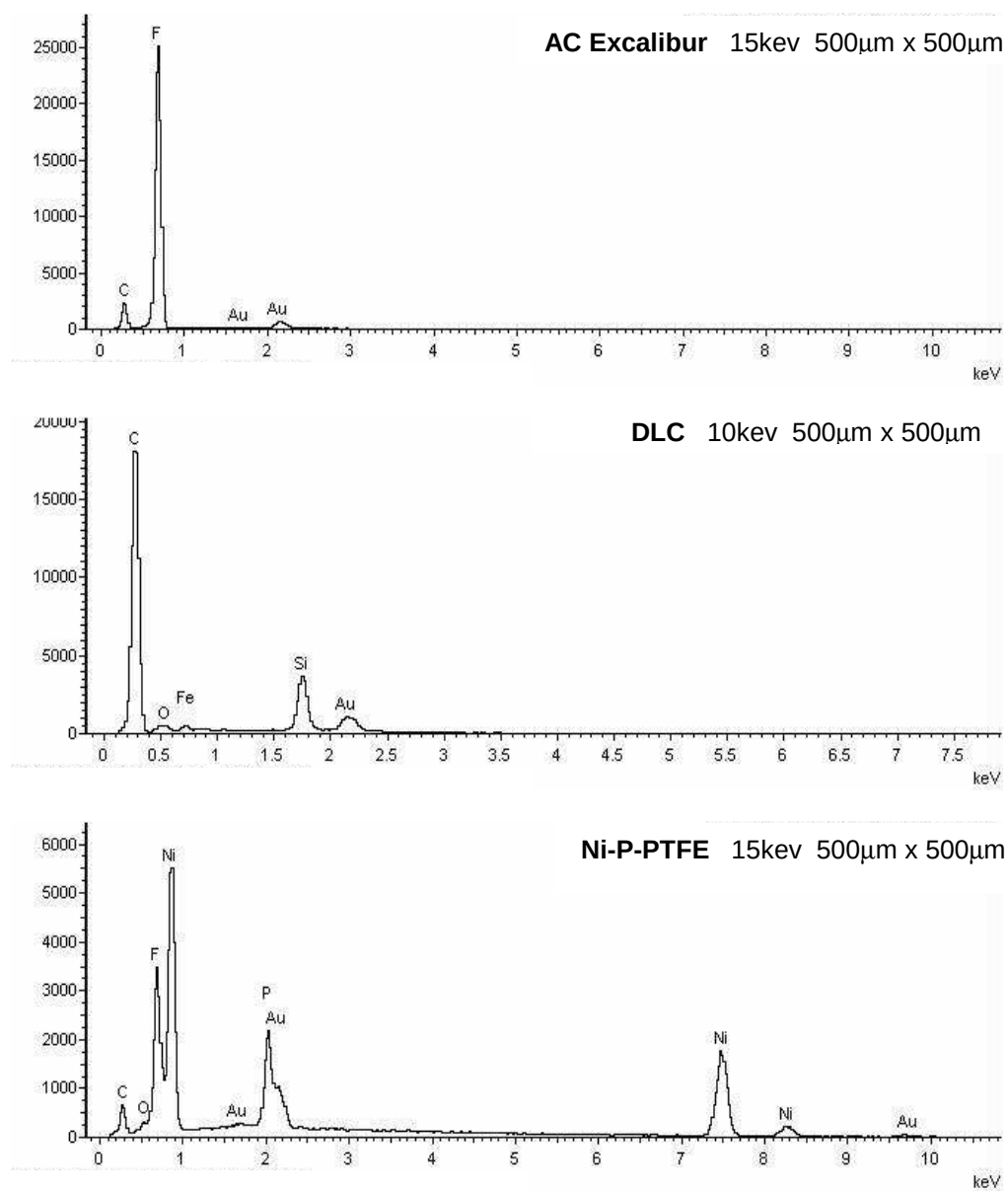


Figure A.7 (cont.) - Spectra of elements obtained by EDS microanalysis with an X-ray energy of 10-15 keV for the different material surfaces.

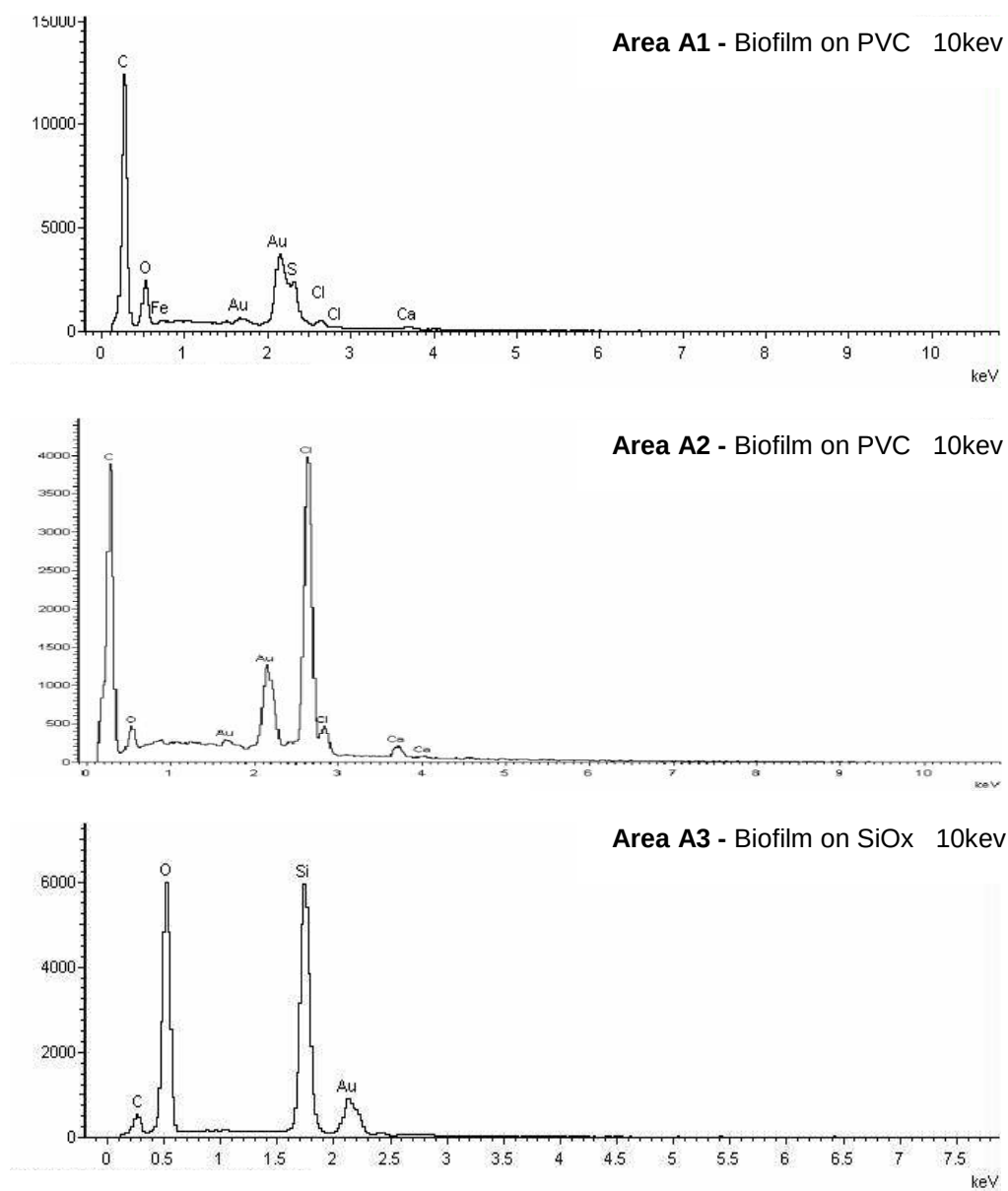


Figure A.8 - Spectra of elements obtained by EDS microanalysis with an X-ray energy of 10-15 keV for the biofilms grown on the different material surfaces.

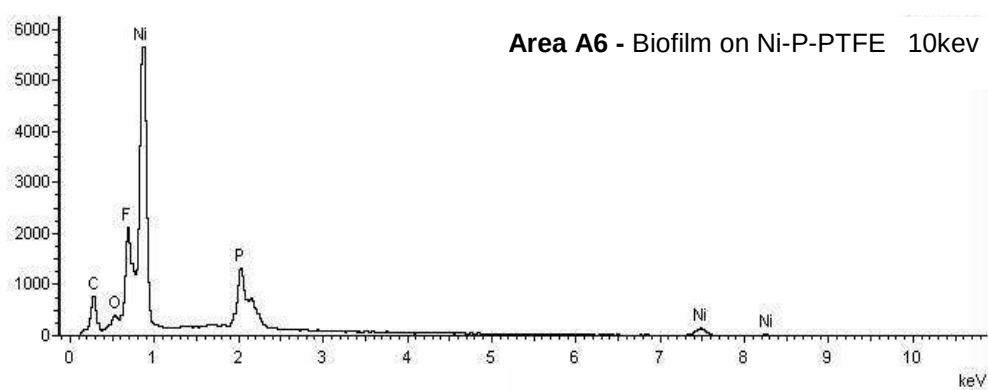
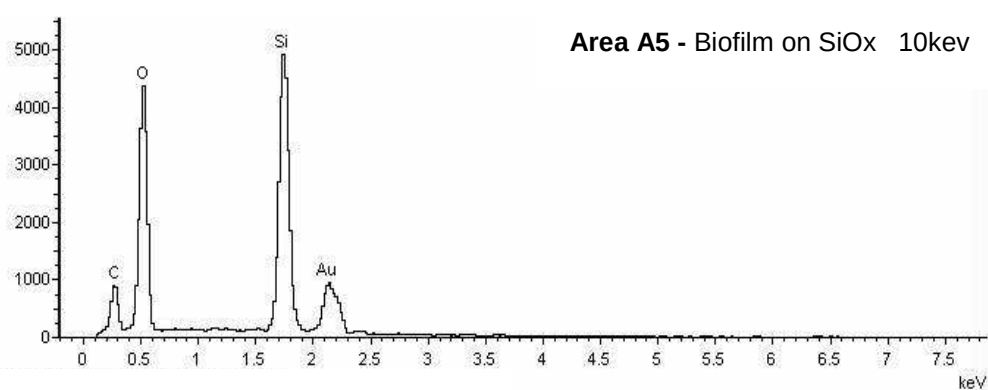
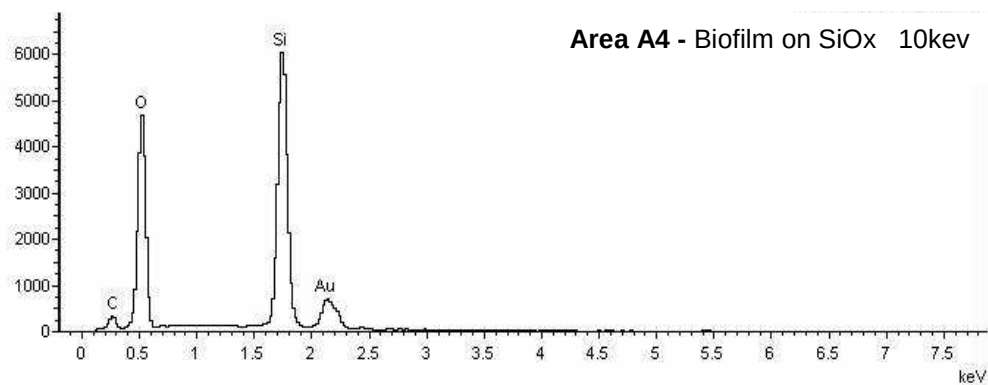


Figure A.8 (cont.) - Spectra of elements obtained by EDS microanalysis with an X-ray energy of 10-15 keV for the biofilms grown on the different material surfaces.

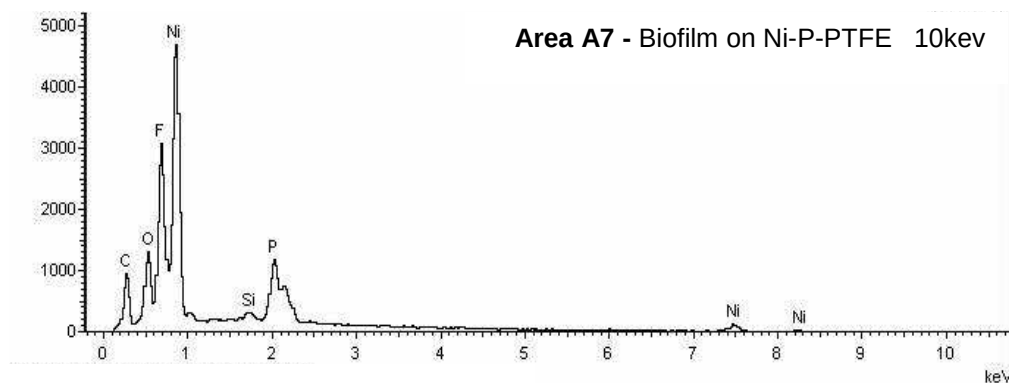


Figure A.8 (cont.) - Spectra of elements obtained by EDS microanalysis with an X-ray energy of 10-15 keV for the biofilms grown on the different material surfaces.

A.10 Principal Components Analysis for the Materials Effect

This statistical tool was performed with the experimental data obtained in **Exp. 6.1**, **Exp. 6.2** and **Exp. 6.3**. The independent parameters used to define the principal components were the following: TC_b - total cells/cm²; MAC_b - fraction of metabolically active cells; CC_b - fraction of cultivable cells present in biofilm; R_a - mean roughness; θ_w - contact angle with water; γ_s^- - negative component of surface free energy; ΔG^{TOT} - total free energy of hydrophobicity.

Firstly, the correlation matrix was determined (Table A.11) which indicated if among the parameters analyzed, two were correlated (when $|r| \geq |r_{crit}|$), independently from the used polymeric materials. The correlation between the variables percentage of metabolically active cells in biofilm and negative component of surface free energy (MAC_b and γ_s^-) was the only that may be considered statistically significant but it is not considered because two of the three materials had the same γ_s^- value. All the other variables were not correlated and thus affected by other non quantified parameters.

Following the PCA analysis, two principal components (PC1 and PC2) were extracted (Table A.11) that explained 91.0 % of the overall variance, being the first one (PC1) that contained the highest variance amount (69.7 %).

The plot of the principal components (Figure A.9) over the original variables of **Exp. 6.1** may indicate those that defined and affected more each PC. All variables seemed to affect similarly PC1 (in absolute values), except the fraction of cultivable cells in the biofilm. This fraction (CC_b) was the variable that affected more PC2.

*Table A.10 - Matrix of the correlations calculated for the variables measured in **Exp. 6.1**. The correlations with statistic significance are indicated in bold (using significance level of 5 %; $|r_{crit}| \geq 0.975$).*

	TC_b	MAC_b	CC_b	Ra	θ_w	γ_s^-	ΔG_{sws}^{TOT}
TC_b	1.000	0.557	0.609	-0.424	-0.863	0.655	0.696
MAC_b	0.557	1.000	-0.194	-0.766	-0.893	0.992	0.907
CC_b	0.609	-0.194	1.000	-0.148	-0.252	-0.097	-0.142
Ra	-0.424	-0.766	-0.148	1.000	0.745	-0.746	-0.467
θ_w	-0.863	-0.893	-0.252	0.745	1.000	-0.938	-0.876
γ_s^-	0.655	0.992	-0.097	-0.746	-0.938	1.000	0.936
ΔG^{TOT}	0.696	0.907	-0.142	-0.467	-0.876	0.936	1.000

TC_b - total cells/cm²; MAC_b - fraction of metabolically active cells; CC_b - fraction of cultivable cells present in biofilm; Ra - mean roughness; θ_w - Contact angle with water; γ_s^- - negative component of surface free energy; ΔG^{TOT} - total free energy of hydrophobicity.

*Table A.11 - Fraction of the total variance explained by each principal component of the data of **Exp 6.1**.*

Principal component	Fraction of the total variance explained (%)	Cumulative fraction of the total variance explained (%)
PC1	69.7	69.7
PC2	21.3	91.0

The plot of the four polymeric materials in the two principal components (Figure A.9) can now indicate how materials may be grouped according to similar biofilm accumulation potential and similar surface properties. Figure A10 shows that HDPE and PP surfaces can be grouped because their PC1 scores were similar (and consequently the variables that define PC1 presented similar values in general) and PC2 scores were close. HDPE had lower PC2 score than the value of PP, due to the higher values of the contact angle with water (θ_w) and the total free energy of hydrophobicity (ΔG^{TOT}). PEX distinguished from the above group by presenting higher total attached cells/cm² and fraction of cultivable cells. PVC stands

completely apart from the others due to the lower contact angle with water and free total energy of hydrophobicity.

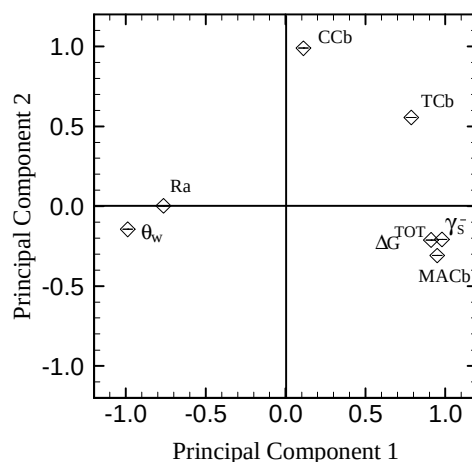


Figure A.9 - Scatter plot of the original variables defined in the first two principal components extracted from data of **Exp. 6.1** that explained 91.0 % of total variance. TC_b - total cells/cm²; MAC_b - fraction of metabolically active cells; CC_b - fraction of cultivable cells present in biofilm; Ra - mean roughness; θ_w - Contact angle with water; γ_s^- - negative component of surface free energy; ΔG^{TOT} - total free energy of hydrophobicity.

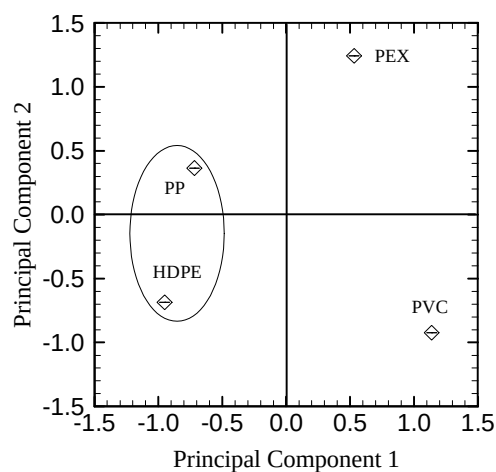


Figure A10 - Scatter plot of the polymeric materials defined in the two principal components that contained 91.0 % of total variance of data in **Exp. 6.1**.

As regards the data of the four polymeric materials of **Exp. 6.2**, the correlation matrix showed that none of the parameters analyzed was correlated (Table A.12). Again, two components PC1 and PC2 were enough to analyze the system since 91.0 % of total variance was described with both (Table A.13).

Table A.12 - Matrix of the correlations calculated for the variables measured in **Exp. 6.2**. The correlations with statistic significance are indicated in bold (using significance level of 5% $|r_{crit}| \geq 0.975$).

	TC _b	MAC _b	CC _b	Ra	θ_w	γ_s^-	ΔG^{TOT}
TC _b	1.000	-0.235	-0.576	0.959	0.888	-0.788	-0.580
MAC _b	-0.235	1.000	-0.260	0.030	-0.568	0.396	0.624
CC _b	-0.576	-0.260	1.000	-0.587	-0.168	-0.042	-0.324
Ra	0.959	0.030	-0.587	1.000	0.783	-0.758	-0.491
θ_w	0.888	-0.568	-0.168	0.783	1.000	-0.938	-0.876
γ_s^-	-0.788	0.396	-0.042	-0.758	-0.938	1.000	0.936
ΔG^{TOT}	-0.580	0.624	-0.324	-0.491	-0.876	0.936	1.000

TC_b - total cells/cm²; MAC_b - fraction of metabolically active cells; CC_b - fraction of cultivable cells present in biofilm; Ra - mean roughness; θ_w - Contact angle with water; γ_s^- - negative component of surface free energy; ΔG^{TOT} - total free energy of hydrophobicity.

Table A.13 - Fraction of the total variance explained by each principal component of the data of **Exp 6.2**.

Principal component	Fraction of the total variance explained (%)	Cumulative fraction of the total variance explained (%)
PC1	63.5	63.5
PC2	27.5	91.0

The representation of the two components with the observed variables and the polymeric materials is presented in Figure A.11. In this case, all analyzed parameters affected similarly (absolute values) the PC1 component, except the fractions of metabolically active and the cultivable cells. These fractions were explained better by the PC2. PEX and PVC were grouped while HDPE and PP were completely apart. HDPE presented high positive values of both PC1 and PC2 which may be attributed to a higher roughness, total attached cells/cm² and contact angle with water. PP had a much lower PC1 score probably due to a high percentage of cultivable cells (CC_b) and low percentage of metabolically active cells in biofilms (MAC_b).

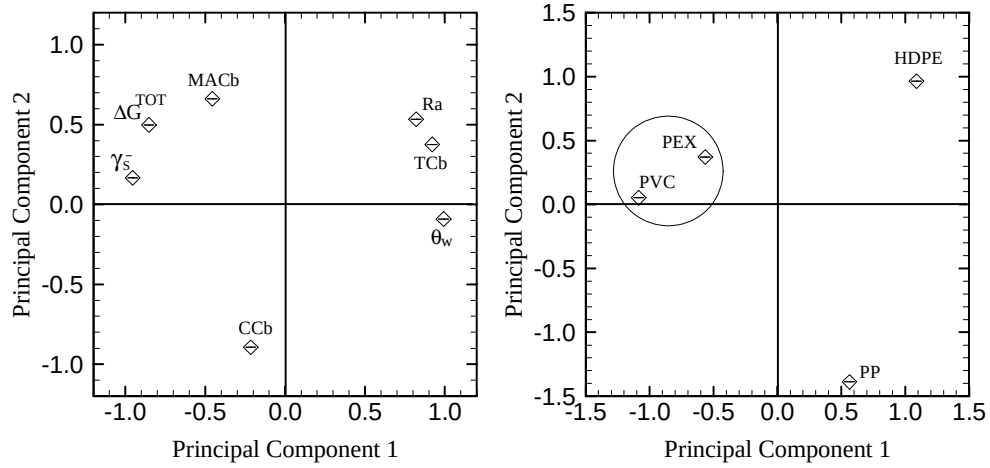


Figure A.11 - Scatter plot of the first two principal components extracted from data of **Exp. 6.2** that explained 91.0 % of total variance. TC_b - total cells/cm²; MAC_b - fraction of metabolically active cells; CC_b - fraction of cultivable cells present in biofilm; Ra - mean roughness; θ_w - Contact angle with water; γ_s^- - negative component of surface free energy; ΔG^{TOT} - total free energy of hydrophobicity.

The output of PCA for the data in Exp. 6.3 is presented in Figure A.12.

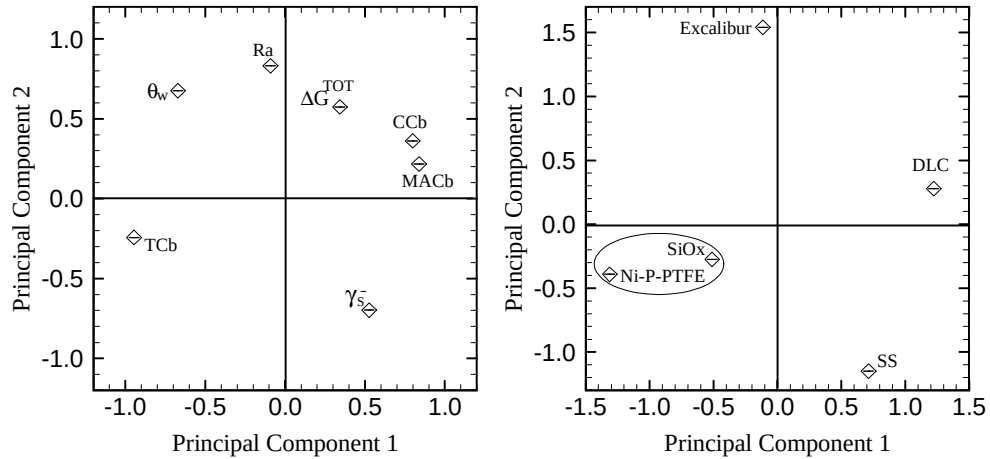


Figure A.12 - Scatter plot of the first two principal components extracted from data of **Exp. 6.3** that explained 75.5 % of total variance. . TC_b - total cells/cm²; MAC_b - fraction of metabolically active cells; CC_b - fraction of cultivable cells present in biofilm; Ra - mean roughness; θ_w - Contact angle with water; γ_s^- - negative component of surface free energy; ΔG^{TOT} - total free energy of hydrophobicity.