Universidade do Minho
Escola de Engenharia

Alexandrina Lima Rodrigues

Influence of Humic Substances on Biofilm Structure and its Microbial Diversity in Natural Waters

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Influence of Humic Substances on Biofilm Structure and its Microbial Diversity in Natural Waters

Doctoral Dissertation for PhD degree in Chemical and Biological Engineering

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Título da tese

Influence of humic substances on biofilm structure and its microbial diversity in natural waters

Influência das substâncias húmicas na estrutura do biofilme e na sua diversidade microbiana em águas naturais

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Rodrigues, A.L., Brito, A.G., Janknecht, P., Proença, M.F., Nogueira, R., Comparison of UV spectroscopy and total organic carbon methods to assess humic substances in surface water. Natural Organic Matter: From Source to Tap (NOM2008), 2nd - 4th September, 2008, Bath, England.

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ABSTRACT

Natural organic matter (NOM) is ubiquitous in terrestrial and aquatic ecosystems; it comprises an important source of carbon for river biofilms which are major sites of carbon cycling in streams. NOM may be classified in two main categories: non-humic and humic substances (HSs). About 75 % of the dissolved organic carbon (DOC) in rivers results from HSs. The presence of HSs in water treatment plants is undesirable because they increase coagulant and disinfectant demands, and in the presence of chlorine may provoke the formation of dangerous disinfection by-products such as the carcinogenic trihalomethanes (THM). A good knowledge of HSs involvement on biofilm growth can contribute to the development of a biological alternative of HSs removal from rivers.

The first step of this work consisted on characterization of HSs and comparison of two methods, combustion-infrared method and UV spectroscopy method, commonly used to HSs quantification in aqueous solution. In this study were evaluated the effect of divalent cations (calcium and magnesium) concentrations, pH and, sample filtration on HSs quantification from simulated river water. A commercial humic acid (HA) was used to simulate HSs. The experimental results demonstrate that unfiltered samples presented considerably higher total organic carbon (TOC) values than filtered ones, independently of the method used. Based on these results, it can be suggested that a considerable amount of HAs was present as colloids with an average diameter higher than 0.45 μm. TOC values of unfiltered samples obtained by both methods were considerably different for all pH values and cation concentrations tested. TOC values obtained by the combustion-infrared method were closer to the concentration of the HAs suspension which was prepared by weighting a defined amount of HAs. The combustion-infrared method gave the most accurate value although the precision was lower than the one obtained in the UV spectroscopy method. In this regard, the combustion-infrared method is recommended for HSs quantifications in surface water where pH values and divalent cation concentration may vary considerably.

Since biofilms are major sites of carbon transformations in rivers, biofilm formation on HSs was studied using simulated river water and model biofilm flowcells to obtain controlled hydrodynamic conditions. Biofilm formation in the presence HSs as a carbon source under a flow velocity of 0.04 m/s was evaluated. Two biofilm flowcells were operated in parallel; one with synthetic stream water, displaying a background carbon concentration of 1.3 ± 0.8 mg/L, the other with added HSs and an

overall carbon concentration of 9.7 ± 1.0 mg/L. From the biofilms' results of culturable and total countable cells, it can be concluded that the presence of HSs did not significantly enhance the biofilm density. However, the biofilm formed in the presence of HSs presented slightly higher values of volatile suspended solids (VSS) and protein. One possible explanation for this result is that HSs adsorbed to the polymeric matrix of biofilm and were included in the quantification of VSS and protein. The bacterial community of biofilms formed with and without HSs was different. In the biofilm with HSs were identified bacteria belonging to beta-Proteobacteria (represented by *Cupriavidus metallidurans* and several species of the genus *Ralstonia*) and gamma-Proteobacteria (represented by *Escherichia coli*). In the biofilm formed without HSs were identified bacteria of groups beta-Proteobacteria (represented by *Variovorax paradoxus*) and Bacteroidetes.

Flow velocity, concentration and nature of carbon source are of great importance on the biofilm development. Thus, in the last work it was investigated the response of biofilms formed with and without HSs to an increase in flow velocity (0.04 to 0.10 m/s) and HSs concentration (9.7 ± 1.0 to 19.8 ± 0.4 mg/L). The highest biofilm density, according to VSS and total countable cells, was detected at 0.10 m/s without HSs. In this regard, organic carbon present in distilled water and in HSs may have not the same bioavailability. HSs concentration had no significant effects on biofilm cell density under different flow velocities. However, HSs presence influenced the biofilm bacteria composition. Sequences retrieved from biofilms formed in the presence of HSs were related (similarities ≥ 97 %) to *Dokdonella* genus which belongs to gamma-Proteobacteria and to *Comamonas*, *Cupriavidus* and *Ralstonia* genera which belong to beta-Proteobacteria. Sequences retrieved from the biofilm without HSs were related (similarities ≥ 97 %) to *Sphingomonas* and *Nitrosospira* genera that belong to alpha-Proteobacteria and beta-Proteobacteria, respectively.

In conclusion, based on this work an increased understanding was gained of HSs effects on biofilm formation under different hydrodynamic conditions, however additional studies are needed to quantify HSs biodegradability in biofilms.

RESUMO

A material orgânica natural (MON) é ubíqua em ecossistemas terrestres e aquáticos e constitui uma fonte de carbono importante para os biofilmes, presentes nos rios, que são os principais responsáveis pelo ciclo do carbono. A MON pode ser classificada em duas categorias principais: substâncias não húmicas e substâncias húmicas (SHs). Nas águas superficiais aproximadamente 75 % do carbono orgânico dissolvido (COD) resulta das SHs. A presença de SHs nas estações de tratamento de água é indesejável porque aumenta a necessidade de adição de coagulantes e desinfectantes e na presença de cloro pode conduzir à formação de subprodutos de desinfecção, nomeadamente, trihalometanos (THM) que são compostos carcinogénicos. Um bom conhecimento da contribuição das SHs no crescimento dos biofilmes pode conduzir ao desenvolvimento de uma alternativa biológica para a remoção das SHs das águas superficiais.

O primeiro passo deste trabalho consistiu na caracterização das SHs e comparação de dois métodos, método combustão e detecção de infravermelhos e método espectrofotométrico, frequentemente utilizados na quantificação de SHs em solução aquosa. Neste estudo foram avaliados os efeitos da concentração dos iões divalentes (cálcio e magnésio), pH e filtração da amostra na quantificação das SHs de água de rio sintética. Foi utilizado ácido húmico (AH) comercial para simular SHs. Os resultados experimentais demonstraram que as amostras não filtradas apresentaram valores de carbono orgânico total (COT) consideravelmente superiores aos valores das amostras filtradas, independentemente do método utilizado. Com base nestes resultados, pode sugerir-se que uma quantidade considerável de AHs estava presente em forma de colóides com um diâmetro, em média, superior a 0.45 μm. Os valores de COT das amostras não filtradas obtidos pelo método de combustão e detecção de infravermelhos estavam mais próximos da concentração dos AHs que foi preparada com a pesagem de uma quantidade definida de AH. O método de combustão e detecção de infravermelhos apresentou o valor mais exacto apesar da precisão ter sido inferior ao valor obtido pelo método espectrofotométrico (UV). Neste sentido, o método de combustão e detecção de infravermelhos é recomendado para a quantificação de SHs em águas superficiais cujos valores de pH e concentração de catiões divalentes podem variar consideravelmente.

Uma vez que os biofilmes são os principais locais de transformação de carbono nos rios, foi estudada a formação de biofilme na presença de SHs utilizando-se água de rio sintética e células de fluxo modelo de modo a obter condições hidrodinâmicas controladas. Foi avaliada a formação de biofilme na presença de SHs como fonte de carbono à velocidade de 0.04 m/s. Foram operadas duas células de fluxo em paralelo; uma com água sintética de rio, contendo uma concentração "background" de carbono de 1.3 ± 0.8 mg/L, outra com a adição de SHs e, geralmente, com concentração de carbono de 9.7 ± 1.0 mg/L. A partir dos resultados das células cultiváveis e das células totais contáveis, pode-se concluir que a presença de SHs não favoreceu significativamente a densidade celular do biofilme. No entanto, o biofilme formado na presença de SHs apresentou valores de sólidos suspensos voláteis (SSV) e de proteína ligeiramente superiores. Uma explicação possível para este resultado consiste no facto das SHs terem adsorbido à matriz polimérica do biofilme e terem sido incluídas na quantificação dos SSV e proteína. A comunidade bacteriana dos biofilmes formados com e sem SHs foi diferente. No biofilme com SHs foram identificadas bactérias que pertencem aos grupos beta-Proteobacteria (representado pela espécie *Cupriavidus metallidurans* e algumas espécies do género *Ralstonia*) e gamma-Proteobacteria (representado pela espécie *Escherichia coli*). No biofilme formado sem SHs foram identificadas bactérias dos grupos beta-Proteobacteria (representado pela espécie *Variovorax paradoxus*) e Bacteroidetes.

A velocidade, concentração e natureza da fonte de carbono têm elevada importância no desenvolvimento do biofilme. Deste modo, no último trabalho foi investigada a resposta dos biofilmes formados com e sem SHs a um aumento de velocidade (0.04 para 0.10 m/s) e concentração de SHs (9.7 ± 1.0 para 19.8 ± 0.4 mg/L). A densidade celular de biofilme mais elevada, de acordo com os resultados dos SSV e células totais contáveis, foi detectada a 0.10 m/s sem SHs. Neste sentido, o carbono orgânico presente na água destilada e nas SHs pode não ter a mesma biodisponibilidade. A concentração de SHs não teve efeitos significativos na densidade celular do biofilme sob diferentes velocidades. No entanto, a presença de SHs influenciou a composição bacteriana do biofilme. Sequências obtidas a partir de biofilmes formados na presença de SHs foram relacionadas (similaridades ≥ 97 %) com o género *Dokdonella* que pertence ao grupo gamma-Proteobacteria e com os géneros *Comamonas*, *Cupriavidus* e *Ralstonia* que pertencem ao grupo beta-Proteobacteria. Sequências obtidas a partir de biofilme sem SHs foram relacionadas (similaridades ≥ 97 %) com os géneros *Sphingomonas* e *Nitrosospira* que pertencem, respectivamente, aos grupos alpha-Proteobacteria e beta-Proteobacteria.

Em conclusão, com base neste estudo, aumentou-se o conhecimento dos efeitos das SHS na formação de biofilmes sob diferentes condições hidrodinâmicas, no entanto são necessários estudos adicionais para quantificar a biodegradabilidade das SHs nos biofilmes.

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LIST OF ABREVIATIONS AND SYMBOLS

CE - Capillary electrophoresis.

CFU – Colony forming units.

DAPI - 4',6-diamino-2-phenylindole.

DGGE – Denaturing of temperature gradient gel electrophoresis.

DOC - Dissolved organic carbon.

DOM - Dissolved organic matter.

DRIFT - Diffuse reflectance infrared fourier transform.

EPA - Environmental protection agency.

EPR - Electron paramagnetic resonance.

EPS – Extracellular polymeric substances.

FAs - Fulvic acids.

FTIR - Fourier transform infrared.

GC – Gas chomatography.

HA - Humic acid.

HAA - Haloacetic acids.

HAs - Humic acids.

HMW - High molecular weight.

HSs – Humic substances.

Hu – Humin.

IHSS - International humic substances society.

IR – Infrared.

LMW – Low molecular weight.

MS – Mass spectrometry.

NMR - Nuclear magnetic resonance.

NOM - Natural organic matter.

PCR – Polymerase chain reaction.

TC – Total countable cells.

THM – Trihalomethanes.

TOC - Total organic carbon.

VSS – Volatile suspended solids.

ZP – Zeta potential.

1 | THESIS OVERVIEW AND GENERAL INTRODUCTION

1.1 CONTEXT AND MOTIVATION

The scarcity of drinking water around the world and increasing requirements concerning its quality foster the development of new effective processes of water treatment. Stream waters are the main source of water for the drinking sector, which are usually associated to high levels of natural organic matter (NOM). NOM is a mixture of organic molecules of diverse structure, molecular weight, complexity and biodegradability. Humic substances (HSs) are the main fraction of NOM and are responsible by the aesthetically undesirable water, but they are considered harmless on its own. However, its interaction with chlorine, widely used to inactivate pathogenic microorganisms during drinking water purification, may result in the formation of harmful disinfection by-products with undesirable human health effects. Trihalomethanes (THM) and haloacetic acids (HAA) are the main disinfection by-products formed upon chlorination of water. They cause cancer in animals, and are human carcinogens (Richardson, 2005). In this regard, the US Environmental Protection Agency (EPA) allows a maximum contaminant level of 80 μg/L (EPA, 1998) and the European Union has established drinking water guideline values for the sum of THM at 100 μg/L (Directive 98/83/EC, 1998).

As a combined consequence of the deterioration in water sources and more strict regulatory requirements for disinfection by-products on drinking water, many NOM laden waters have become more difficult to treat using traditional techniques in order to comply with current legislation (Eikkebrokk *et al.*, 2004). Furthermore, recent issues have arisen for water utilities around the increased costs of coagulants (linked to increased transport costs) and security of supply. For these reasons alternative and additional treatment stages have been investigated (Singer and Bilyk, 2002; Parsons *et al.*, 2007). In aquatic ecosystems, most microbes live in matrix enclosed biofilms (Costerton *et al.*, 1995; Palmer and White, 1997; Sutherland, 2001a) and contribute substantially to dissolved organic carbon cycling, including humic substances (Seitzinger *et al.*, 2005; Frazier *et al.*, 2005; Kim *et al.*, 2006). Biofilms are important components of food chains, and are involved in self-purification in stream waters (Lawrence and Neu, 2004; Denkaus *et al.*, 2007). Therefore, stream biofilms application could represent a strategy to improve surface water quality before chemical treatment reducing the demand of chlorine.

1.2 OBJECTIVES AND OUTLINES

The work presented in this thesis deals with humic substances (HSs) characterization and quantification in surface waters as well as HSs influence on biofilm formation and its bacterial composition in simulated river water conditions. In this regard, flowcells were designed in order to obtain controlled hydrodynamic conditions.

The aim of this work was to characterize and quantify HSs as well as to study its effects on biofilms growth in simulated river water conditions. Therefore was explored biofilms capacity for surface waters purification.

This thesis is organized in five Chapters. Firstly, in Chapter 1 is presented the context and motivation of the work, as well as its main objectives. Also, chemical composition, characterization, quantification and biodegradability of HSs in natural systems as well as the factors that influence biofilms growth are presented. Chapter 2 compares two methods, absorbance of ultraviolet light at 254 nm (UV254) and total organic carbon (TOC) (combustion-infrared method) to assess humic acids (HAs) in surface water and presents the effects of calcium and magnesium concentrations, pH and sample filtration on the methods' results. Chapter 3 describes the biofilm formation with and without HSs. Chapter 4 deals with the response of biofilms formed with and without HSs to an increase in flow velocity and HSs concentrations. Finally, in Chapter 5 conclusions and suggestions for future work are presented.

1.3 HUMIC SUBSTANCES

1.3.1 Humic Substances Chemical Composition

Humic substances (HSs) constitute the main group of NOM that result from decomposition of terrestrial and aquatic biomass and can not be exactly classified as any other chemical class of compounds (e.g. polysaccharides, proteins, lignin, etc) (Steelink *et al.*, 1999; Xing *et al.*, 1998). The HSs are not made up of discrete, well-defined molecules but are a class of substances that are produced and reside in soil and water (vanLoon and Duffy, 2005). The residues of organic matter produced on soils are available to be transferred into the hydrosphere. In surface water, HSs comprise 50-75 % of the dissolved organic carbon (DOC) which concentrations range from 1 to 60 mg/L (Amon and Benner, 1996; Hertkorn *et al.*, 2002a). The size, molecular weight, elemental compositions, structure, and the number and position of functional groups of HSs vary depending on their origin, method of extraction, and natural condition which prevailed on their formation (Balnois *et al.*, 1999; Hayes, 1998). Individual molecules can not be identified from HSs (also called humic material, humate or humus), but they might be subdivided into three fractions according to their solubility in water: humic acids (HAs) that are insoluble under acid conditions (pH2) but soluble at elevated pH, fulvic acids (FAs) that are the fraction of HSs soluble in aqueous solutions under all pH values, and humin (Hu) that is insoluble in water at all pH values (Stenveson, 1982; Magdaleno and Coichev, 2005; Kim and Osako, 2004).

HSs formation is poorly understood, although many studies have been carried out in order to improve knowledge about this issue (Kononova, 1966; Schnitzer and Khan, 1978; Stevenson, 1982, Stevenson, 1994, Qualls *et al.*, 2003; Kudeyarova, 2006; Slawiñska *et al.*, 2007). There are several pathways for the formation of HSs during the decay of plant and animal remains in soil, the main four ones are depicted in the Figure 1.1.

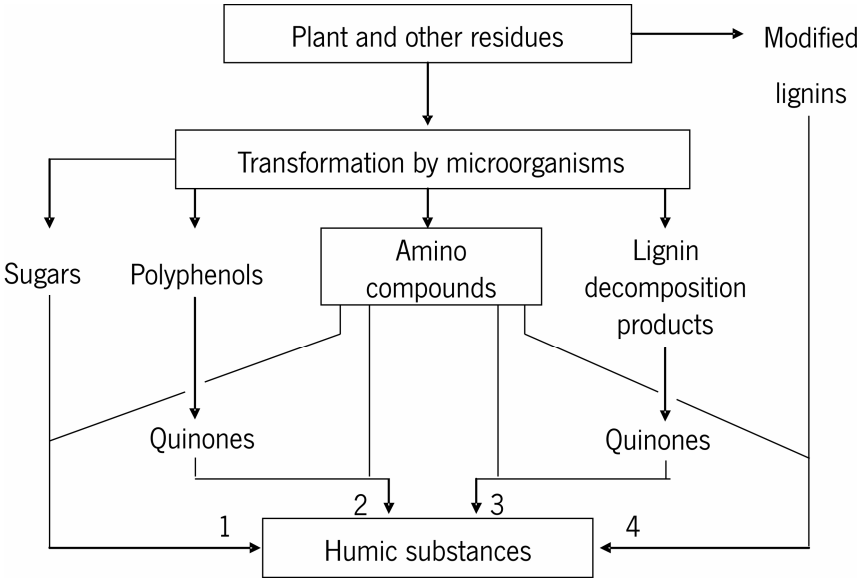

Figure 1.1 Pathways for the formation of soil HSs (Stenveson, 1994).

These four pathways may operate simultaneously in all soils, but not at the same extent or in the same order of importance. The concept that HSs are formed from sugars (pathway 1) dates back to the early days of HSs chemistry. This pathway suggests the formation of HSs as a result of the condensation of sugar and amino acids, formed as by-products of microbial metabolism, under nonenzymatic polymerization. Alternatively, HSs may be formed by processes that involve quinones (pathways 2 and 3) or may represent modified lignins (pathway 4). A lignin pathway may prevail in poorly drained soils and wet sediments, whereas synthesis from quinones (from polyphenols oxidation) may be of considerable importance in several forest soils. The pathway sugar-amine condensation is valid for situations with low microbial activity. However, Malcolm (1990) stated that lignin is not the main precursor of soil HSs, in fact he has shown that there is a great structural difference between HSs from soils, rivers and sea. Pathways 2 and 3 based on polymeric condensation of polyphenols and quinones have been the more accepted mechanisms of HSs formation by most researchers and by the International Humic Substances Society (IHSS) (Stenveson, 1994).

VanLoon and Duffy (2005) described the formation of HSs by two only pathways. The first one is a degradative pathway where labile macromolecules such as carbohydrates and proteins are degraded and lost during microbial attack, while refractory compounds or biopolymers (e.g. lignin, paraffinic macromolecules, melanins, and cutin) are selectively transformed to produce a high molecular mass

precursor of humin. Additional oxidation of these materials generates increased oxygen content in the form of typical functional groups like carboxylic acids and, as this process continues, the molecules become small enough and hydrophilic enough to be soluble in alkali. In the end, the molecules may become even smaller and sufficiently oxygen-rich to dissolve in both acid and base. The wide degradation produces structures that retain some original features but also have substantial structural dissimilarity from the parent material. The second one is a synthetic pathway where plant biopolymers are initially degraded to small molecules after which these molecules are repolymerized to form HSs. It has been suggested that polyphenols synthesized by fungi and other microorganisms, together with those released from the oxidative degradation of lignin, undergo oxidative polymerization. A result of this proposal is that fulvic acid would be a precursor of humic acid and then of humin (the reverse of the degradative theory). This hypothesis may explain the observed large similarity of HSs formed from a diversity of precursor macromolecules in different environments. The two suggested pathways have overlapping features and both ways could improve the knowledge to the actual formation of HSs depending on the environment. For example, the degradative pathway might prevail in wet sediments and in the aquatic environment in most cases, whereas the conditions in soils with typical continental climates might favour a condensation polimerization scheme.

The analysis of a wide variety of HSs shows that they usually contain carbon, hydrogen, oxygen, and nitrogen within ranges as described in Table 1.1 (Stenveson, 1994; Kurková *et al.*, 2004).

Table 1.1 Usual ranges for the elemental composition of HSs.

Element	Humic acids (%)	Fulvic acids (%)
Carbon	53.8 - 58.7	40.7 - 50.6
Oxygen	32.8 - 38.3	39.7 - 79.8
Hydrogen	3.2 - 6.2	3.8 - 7.0
Nitrogen	0.8 - 4.3	0.9 - 3.3
Sulfur	0.1 - 1.5	0.1 - 3.6

The carbon content of HSs from a given source usually increases in the series: FAs < HAs < Hu. The oxygen content of the same fractions of humic substances follows the reverse trend. The presence of oxygen and nitrogen in HSs is an indication that certain functional groups are present in their

molecules. The most important functional groups that have been reported in HSs include carboxyl (COOH), phenolic (OH), enolic (OH), quinone, hydroxyquinone, lactone, ether, and alcoholic (OH). The functional groups are responsible for important reactions in HSs:

- These groups, individually or together, enable specific reaction of humic substances with inorganic elements and with other organic molecules in soil-water systems.
- The functional groups are also major contributors to the ion exchange properties. Oxygen and nitrogen present in the functional groups behave as bridging units.

Although the precise structure of HAs and FAs is unachievable, the knowledge of the basic structure is required for a better understanding of the properties and function of these constituents in the environment (Avena *et al.*, 1999; Alvarez-Puebla *et al.*, 2006). The main advantages of the hypothetical models consist of their contribution for: (a) the formulation of new hypotheses regarding their structures and the development of innovative experimental schemes for the investigation, and (b) the explanation of the mechanisms of the binding of metal ions and other compounds (Stenveson, 1994). The hypothetical structures of HAs and FAs are depicted in Figures 1.2 and 1.3 (Buffle *et al.*, 1977; Stenveson, 1994).

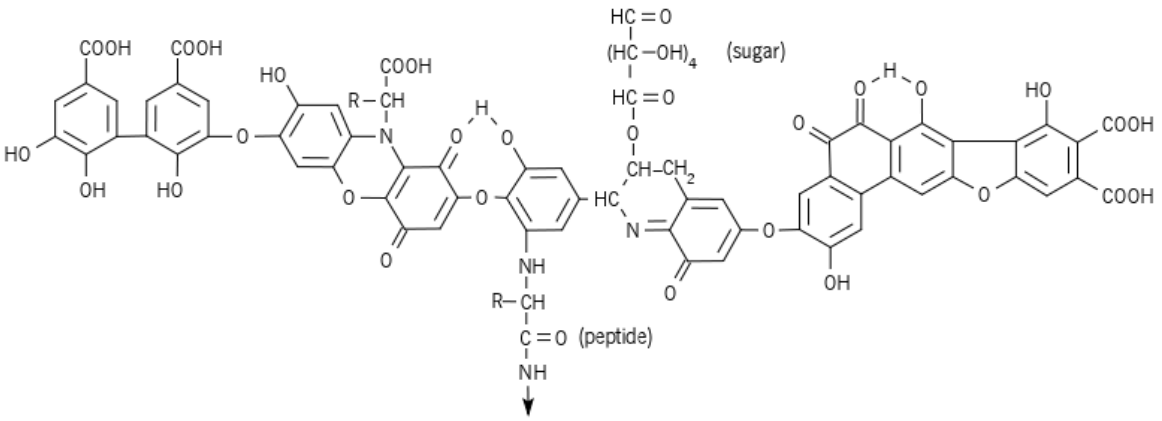

Figure 1.2 Model structure of HAs (Stenveson, 1982).

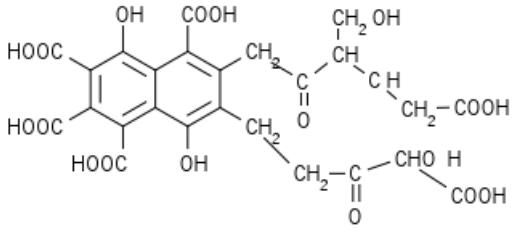

Figure 1.3 Model structure of FAs (Buffle *et al.*, 1977).

Humic substances (HSs) have a wide range of molecular weights and sizes, ranging from a few hundred to as much as several hundred thousands atomic mass units (Da). In most cases, FAs have lower molecular weight than HAs, and soil-obtained materials are larger than aquatic materials (Tombácz *et al.*, 1990). The structures of FAs are somewhat more aliphatic and less aromatic than HAs, and FAs are richer in carboxylic acid, phenolic and ketonic groups (Hosse and Wilkinson, 2001; Lead *et al.*, 2000). This is responsible for the higher solubility of FAs in water at all pH. HAs, being more aromatic, become insoluble when the carboxylated groups are protonated at low pH values. This structure allows HSs to behave like amphiphilic compounds, with the ability to bind both hydrophobic and hydrophilic materials (Schulten and Schnitzer, 1995; Pompe *et al.*, 1996).

The conceptual model of HSs conformation has developped over the past decade. The traditional theory is that HSs are large polymers and may occur in linear or coiled, crosslinked conformations, depending on the properties of the solution. According to this model, at high concentration, low pH, and high ionic strength these molecules exist in the coiled conformation, while they exist as flexible linear polymers at neutral pH, low ionic strength, and low concentration (Stenvenson, 1994). Another theory proposes that HSs in solution are a loosely bound self-association of somewhat small molecules which are dominated by intermolecular hydrophobic interactions as binding forces (Conte and Piccolo, 1999). In aqueous environments, HSs assume extended shapes due to intramolecular electrostatic repulsive interactions. The hydrophobic effect causes the exterior to be hydrophilic and the interior to be hydrophobic (Stumm and Morgan, 1981).

1.3.2 Humic Substances Interaction with Metal Ions

The chemical behaviour of HSs in aqueous solution is not fully understood even though considerable advances have been made over the past decade. This lack of knowledge is a consequence of a combination of factors such as (1) the absence of a unique structure of HSs, (2) the variation in the methods of isolation and extraction, and (3) the absence of specific analytical methods suitable only for HSs. Humic substances reactivity in the environment depends on their functional group chemistry and macromolecular structure, their shape and size. Therefore, changes in HSs macromolecular structure are important for their geochemical reactions: they play a crucial role in the transport, bioavailability and deposition of several heavy metal ions (Linnik, 2003; Chakraborty and Chakrabarti, 2008). Among the HSs, the humic acids (HAs) and fulvic acids (FAs) are polydisperse mixtures of natural organic polyelectrolytes having different types of functional groups to which ions can bind. Two major types of functional groups are of particular importance, namely,

carboxylic groups and phenolic groups (Perdue, 1985; De Wit 1993a; De Wit 1993b; Kantar and Karadağli, 2005). Amino, sulfhydryl, and quinine groups can also be present and may have strong interactions with trace metal ions (Perdue, 1985; Aiken *et al.*, 1996). However, since their number is much smaller, their contribution to ion binding is also generally small, although under certain conditions it can be substantial.

There are investigations which reported that aqueous solutions of soil HAs responded to the addition of cations forming compact structures with reasonably hydrophobic interiors and hydrophilic surfaces (Wandruszka *et al.*, 1997; Palmer and von Wandruszka, 2001; Anđelković *et al.*, 2004). After the addition of cations, HAs macromolecules tend to shrink or contract; mutual repulsion among negatively charged carboxyl groups is minimized and they fold forming intramolecular and intermolecular aggregates. This could be a consequence due to two mechanisms: charge neutralization and functional group bridging. Functional group bridging increases this effect, particularly with multivalent cations, by drawing together various groups on the humic acid chain (Tripathy and Ranjan De, 2006). Natural waters contain a variety of ions, namely calcium (Ca^{2+}) and magnesium (Mg^{2+}), that will compete to a greater or a lesser extent for the available binding sites of HAs (Buffle, 1988; Ahn *et al.*, 2008). Protons are always present in aquatic systems, and they too will compete with metal ions for binding to HAs. The proton concentration determines the degree of ionization of the functional groups as well as the charge of the HAs. Therefore pH is one of the variables that should be considered in complexation reactions, because it affects the surface charge of the humic molecules, and thereby also affects their affinity for metals. The charge state of these molecules can be characterized by electrophoretic mobility or zeta potential (Yoon *et al.*, 1998; Alvarez-Puebla and Garrido, 2005; Majzik and Tombácz, 2007).

1.3.3 Methods of Humic Substances Characterization and Quantification

Humic substances (HSs) in surface waters can be at the origin of various problems in drinking water quality. HSs can be responsible for water taste, odor, and colour. Moreover, these substances also lead to the formation of disinfection by-products after reacting with chloride during water treatment. Therefore, the characterization and quantification of HSs of the stream waters, which are the main water suppliers to water treatment plants, becomes very important.

Humic substances are made up of thousands of diverse molecules making it impractical to separate out each molecule for analysis. Therefore, the characterization of a complex mixture such as HSs

results in values representing an average for the entire mixture. Almost every analytical technique available has been used in an attempt to characterize the complex properties and characteristics of HSs, however, there is no particular analytical method or combination of methods that may provide complete data for total characterization of the structure of HSs. Due to the great heterogeneity of HSs, modern analytical methods are continually being improved in order to obtain reliable results. Besides, since HSs in stream waters are quite low, powerful techniques with high sensitivity are needed (Stenson *et al.*, 2002; Stenson *et al.*, 2003).

The methods of characterization of HSs according to the information provided may be grouped into four main categories: (1) elemental and molecular: elemental composition; (2) functional group: infrared (IR), Fourier transform infrared (FTIR), diffuse reflectance infrared fourier transform spectroscopy (DRIFT), nuclear magnetic resonance (NMR) and Raman spectroscopy, electrometric titrations; (3) mass/size/charge: capillary electrophoresis (CE), gel chromatography, reverse phase chromatography (RP-C18-separation), mass spectrometry (MS), microscopy and viscometry; and (4) other: spectroscopy – UV-visible, fluorescence, electron paramagnetic resonance (EPR) and thermal analysis (Abbt-Braun *et al.*, 2004; Dittmar and Kattner, 2003; Peuravuori *et al.*, 2005; Alvarez-Puebla *et al.*, 2006). The most used methods are described in Table 1.2.

The elemental composition corresponds to the most fundamental characteristic of organic compounds and might be determined with good precision (Huffman and Stuber, 1985). The ash content, calculated by the difference (100 % - C, H, O, N, S), indicates the kind of isolation process. The content of C, H, O, N, and S provides crucial information on the origin of the sample. The H to C ratio (H/C) is an indicator of the extent of saturation of C atoms and/or branched structures contained by the molecule. The lower the H/C ratio, the higher the amount of unsaturated structures. The O to C ratio (O/C) is an indicator of the degree of oxidation and carbohydrate content. N/C values are more elevated for waters with higher microbial dominance than plant material.

Table 1.2 Common methods for the characterization of HSs (Abbt-Braun *et al.*, 2004).

Method		Information	References
Chemical / physical	elemental analysis	elemental composition (C, H, N, O, S)	Hufffman and Stuber, 1985; Abbt-Braun and Frimmel, 2002
	derivatization (methylation, silylation)	functional groups (-OR, -OOR)	Schnitzer and Khan, 1978; Stevenson, 1982
	acid / base-titration		Barak and Chen, 1992; De Wit *et al.* , 1993; Perdue, 1998; Abbt-Braun and Frimmel, 2002
	thermal degradation (pyrolysis)	degradation products (monomers), in combination with GC/MS, LC/MS, or MS	Bracewell *et al.* , 1989; Abbt-Braun *et al.* , 1989 Schulten *et al.* , 2002
	oxidative, reductive degradation	degradation products (monomers), in combination with GC/MS or LC/MS	Schnitzer, 1972; Christman *et al.* , 1989; Parson, 1989
	microscopy	size, shape	Chen and Schnitzer, 1989
	viscometry	size, shape	Clapp *et al.* , 1989
Spectroscopy	UV/VIS	determination of UV/Vis-absorbing groups, (A(254 nm) and A(436 nm); E4/E6	Stevenson, 1982; Hayes *et al.* , 1989; Abbt-Braun and Frimmel, 2002
	IR, FTIR, DRIFT	qualitative determination of functional groups	Hayes *et al.* , 1989; Abbt-Braun, 1992; Niemeyer *et al.* , 1992
	fluorescence	qualitative determination of fluorescent groups	Senesi, 1990; Senesi *et al.* , 1991; Kumke *et al.* , 1998 a
	NMR (^{1}H, ^{13}C)	quantitative determination of functional groups: aromatics, aliphatics, carbohydrates	Wilson, 1987; Frimmel *et al.* , 2002
	NMR (2-D, ^{1}H, ^{13}C)	qualitative / quantitative determination of monomeric units and substitution pattern	Lambert *et al.* , 1992; Hertkorn *et al.* , 2001
	NMR (^{15}N)	qualitative determination of structural elements	Knicker *et al.* , 1997
	Raman	qualitative determination of functional groups	Bloom and Leenheer, 1989
	EPR	quantitative determination of the organic radicals and paramagnetic transition metals	Senesi and Steelink, 1989
	MS	determination of organic molecules; in combination with other methods (e.g. chemical/physical degradation methods, pyrolisis-GC/MS hydrolysis-GC/MS, oxidation-GC/MS)	Schulten *et al.* , 2002; Parson, 1989; Christman *et al.* , 1989
Chromatography	CE; gel chromatography	molecular size, weight	Perminova *et al.* , 1998; Schmitt-Kopplin *et al.* , 1998; 2002 DeNobili and Chen, 1999; Abbt-Braun and Frimmel, 2002
	RP-C18-separation	hydrophobicity	Gremm *et al.* , 1991
Model reactions in combination with the methods above (selection)			
Fluorescence quenching		interaction with organic micropollutants (PAC, pesticides), metals	Karickhoff, 1984, Weber, 1988; Kumke *et al.* , 1998b; Senesi, 1990; Frimmel *et al.* , 2002
Chemical / enzimatic hydrolysis		determination of amino acids, short chain acids, monosaccharides	Jahnel *et al.* , 1998; 2002; Frimmel *et al.* , 2002
Biological degradation		biological utilization	Frimmel *et al.* , 2002
Adsorption behavior		adsorption on solid phases (e.g. activated carbon, mineral phases)	Abbt-Braun *et al.* , 1994 Frimmel and Huber, 1996; Frimmel *et al.* , 2002 Schmitt *et al.* , 2002

Although the elemental composition is an important method of HSs characterization, information about structure and functionality is not enough due to the complexity of these substances. Therefore, a combination of several techniques, with the comparison and confirmation of results from each method, are used to generate a more comprehensive understanding of HSs structure. FTIR, NMR and UV-Vis spectroscopy are also frequently applied methods to the characterization of HSs. FTIR has several advantages over traditional IR techniques. FTIR attains a better sensitivity with increased energy throughput and a higher signal-to-noise ratio. IR spectra are relatively easy to obtain and a large amount of results have been published using FTIR. Therefore, FTIR spectroscopy becomes an attractive technique for the investigation of functional groups in HSs (Davis *et al.*, 1999). However, most studies employ KBr pressed pellets making it difficult to eliminate the interference of water bands in the HSs spectra due to their hygroscopic properties (Tanaka *et al.*, 2001). Also, under specific conditions, KBr has been reported to catalyze the decarboxilation of acidic functional groups, thus altering the HSs (Tanaka *et al.*, 2001).

NMR spectroscopy has been widely applied in the study of HSs (Dria *et al.*, 2002; Chen, 2003; Conte *et al.*, 2004; Cook, 2004; Kim and Yu, 2005; Kelleher and Simpson, 2006). Although specific compound identification is not achievable with this technique, information may be provided about bonding modes of H, C, N and P. Peaks in the NMR spectrum can be assigned to specific functional groups in HSs, however, some overlap of resonance assignments may occur. Despite this, NMR techniques have been valuable in improving the understanding of HSs. A major effort in the characterization of aqueous HSs was made during the ROSIG project using ^{1}H NMR, ^{13}C NMR, ^{15}N NMR, and 2-D NMR techniques for the same set of reference samples (Haiber *et al.*, 2002; Hertkorn *et al.*, 2002b; Lambert and Lankes, 2002; Lankes and Lüdemann, 2002). Among these techniques ^{13}C NMR and ^{1}H NMR are the most used for HSs characterization. ^{13}C NMR is more desirable for elucidating structural information, such as aliphatic, O-alkyl, aromatic and carboxyl carbons, because it examines the actual carbon skeleton rather than protons (Hatcher *et al.*, 1980; Lobartini and Tan, 1988). However the lack of availability of sufficient quantities of HSs samples often prevents application of the ^{13}C NMR technique (Grasso *et al.*, 1990). Analysis by ^{1}H NMR has been shown to exhibit different molecular structures (Grasso *et al.*, 1990), but compared to ^{13}C NMR presents less information because quartenary carbons (e.g. carboxylate groups) cannot be detected (Herzog *et al.*, 1997).

The quatification of HSs in water is not directly possible using common analytical methods due to their complex chemical structure. Therefore indirect methods such as colour, chemiluminescence spectroscopy, UV-vis absorption and total organic carbon (TOC) provide an indication of the total HSs content (Volk *et al.*, 2002; McDonald *et al.*, 2004). Colour measurements provide a simple index to quantify the concentration of HSs in natural waters (Cuthbert and Giorgio, 1992). The chemiluminescence is a spectroscopic method for the quantification of HSs in natural waters which is based upon the oxidation reaction between HSs and chemicals (Michalowski *et al.*, 2001). Absorbance in the UV and Vis range as well as TOC are used widely as group parameters to represent the concentration of HSs in water (Najm *et al.*, 1994; Eaton, 1995). The absorption spectra show a gradual decrease of absorbance from the wavelength λ = 200 nm up to 800 nm. The resolution obtained in such spectra is poor. For HSs quantification, the wavelengths λ = 254 nm (UV) and the yellow colour representing λ = 436 nm (VIS) are used (Chen *et al.*, 1977; Langhals *et al.*, 2000). The absorbance of ultraviolet light at 254 nm is representative of the aromatic compounds present in HSs (Buffle, 1988; Korshin *et al.*, 1997; Uyguner and Bekbolet, 2005). When the absorption characteristics are stable, the UV light absorbance of HSs at a specific wavelength is proportional to their concentration according to the Beer's law. Once the light passes through a water body which contains different constituents of humic substances, the light will be absorbed by the organic compounds in the water and provokes a reduction in the strength of the light. The absorbance depends on the concentrations of the specific compounds and may be used to measure the concentration of the compounds in the solution. From Beer's law, the absorbance of an absorbing specie at a specific wavelength is proportional to the concentration of the compound according to the equation (Skoog and Leary, 1992):

$$A = abc$$

A is the absorbance, *a* is the absorptivity of the analyte, *b* is the optical path length, and *c* is the concentration of the analyte.The Beer's law can also be applied for a solution containing multiple compounds, in which the total absorbance of the multisolute solution at a specific wavelength is equal to the summation of the absorbance of each compound in the solution measured at that wavelength:

$$A = a_1bc_1 + a_2bc_2 + a_3bc_3 + \ldots$$

However, this equation can only be valid when all of the constituents in the solution do not have chemical reaction between each other, namely molecular aggregations. The spectrophotometric method has the potential to discriminate between dissolved organic matter (DOM) fractions, particularly to differentiate the carbon according with their rearrange on the molecular chemical structure. This method only needs a cheaper spectrophotometer compared to a TOC analyzer and the maintenance and operation are simple. The only pretreatment necessary is filtration. The analysis time is short, about one minute for each sample (Skoog and Leary, 1992).

Total organic carbon (TOC) content is a standard method that also has been widely used for monitoring NOM in surface waters (Tipping *et al.*, 1997; Baker, 2002; Wallace *et al.*, 2002). TOC analyzers measure the level of organic carbon amenable to oxidation to carbon dioxide (CO_2). The amount of CO_2 produced may be measured using non-dispersive infrared absorption or conductivity (APHA, 1995). Most waters contain carbonates and bicarbonates, therefore, the concentrations of those inorganic compounds are either measured or the samples are acidified and purged with an inert gas to remove the inorganic carbon (IC) before TOC determination. The TOC method is able to monitor organic carbon independently of their molecular configuration, therefore the chemical reaction between constituents has less influence in this results accuracy than the same results obtained by spectrophotometer method. On the other hand, TOC analyses are associated to a more expansive instrument, a complex operation and higher maintenance costs than the spectrophotometer method.

1.3.4 Humic Substances Biodegradability

Dissolved organic matter (DOM) in aquatic environments represents one of the largest active organic carbon reservoirs (Amon and Benner, 1996). It is a resource that can affect food webs either directly (by uptake from organisms) or indirectly (by mechanisms such as pH, metal binding, and transport of contaminants) (Wetzel *et al.*, 1995a; Guo *et al.*, 1997; Frimmel, 1998; Shetty *et al.*, 2003; Långmark *et al.*, 2004; Alvarez-Puebla *et al.*, 2006; Dong *et al.*, 2006). In most natural waters, the major portion of dissolved organic carbon (DOC) is dominated by humic substances. The bioavailability of DOC to heterotrophic bacteria is likely a consequence of its source, chemical composition, arrangement of the various functional groups, and the ability to react with other chemicals (Saunders, 1976; Münster, 1999). Traditionally, it has been accepted that most of the DOC transformations were accomplished via the metabolism of a small pool of labile, low molecular

weight (LMW) and structurally simple compounds within the DOC (Hertkorn *et al.*, 2002a; Robertson *et al.*, 1999, Hopkinson Jr. *et al.*, 2002; Aminot and Kerouel, 2004). In fact, the physical size of DOC is an important factor influencing microbial utilization, and attempts have been made to classify DOC along a continuum of decreased biological reactivity (Nagata and Kirchman, 1999). Amon and Benner (1996) proposed the size-reactivity continuum model relating the physical size of organic matter to its diagenetic state, suggesting a decrease in size wih increasing diagenesis and chemical alteration. According to this model, organic matter reactivity decreases with decreasing size from high molecular weight (HMW) to LMW and diagenetic state from fresh to old. There is increasing evidence to suggest that the significant fraction of DOC composed of HMW compounds are utilized (Amon and Benner, 1996; Wetzel, 1995b; De Haan, 1977; Benner *et al.*, 1992; Tulonen *et al.*, 1992; Bronk, 1999). Amon and Benner (1996) observed, in fact, that the reactive pool of HMW DOC was larger than the reactive pool of LMW DOC.

Despite the evidence to support the utilization of both LMW and HMW components of DOC, current ideology promulgates the view that only a fraction of the DOC present in stream waters supports bacterial growth. The chemical characteristics of DOC may therefore be important in determining what parts of the DOC are available for utilization. Sun *et al.* (1997) found that the bioavalability of DOC is closely related to the percentage of aliphatic carbon in riverine DOC. Recent works of Fischer *et al.* (2002) and Axmanovà *et al.* (2006) also observed that certain fractions of DOC can be readily utilized for bacterial growth irrespective of molecular size depending on the presence and abundance of specific chemical groups. Straight-chain aliphatic hydrocarbon structures are readily broken down by microorganisms via oxidation processes, but branched and cyclic aliphatic structures, such as terpenoids and steroids, are refractory to aerobic degradation processes (Spitzy and Leenheer, 1991; Greenwood *et al.*, 2006).

There has also been much debate over the biodegradability of humic substances due to their complexity and heterogeneity (Hertkorn *et al.*, 2002a), and they are usually associated with being refractory or recalcitrant. In part this is due to their ability to form complexes with extracelullar enzymes and thus competitively and non-competitively inhibit their activity (Wetzel, 1992). There are exceptions to this thought, however. There is growing evidence to suggest that humic substances are in fact susceptible to microbial degradation. Interestingly, in one experiment, humic acids were more bioavailable than fulvic acids which challenges the generally accepted view that higher molecular weight substances are more refractory than low molecular weight substances (Boyer and Groffman,

1996). De Haan (1974) suggested that a Pseudomonas species could co-metabolize fulvic acids via the mechanism used for lactic acid oxidation. This same researcher used benzoic acid addition in water to stimulate bacterial growth, which in turn lead to the disappearance of colour contributed by fulvic acids (De Haan, 1977). Scott *et al.* (1999) concluded that bacteria can use quinone groups of humic substances as electron acceptors and/or shuttles for anaerobic Fe(III)-reduction. Bano *et al.* (1997) studied the bacterial utilization of dissolved humic substances from a freshwater wetland and observed that suspended bacterial growth efficiency on humic substances was approximately 22 %. There are several works that suggested that biofilm bacteria are also capable of using humic materials (Volk *et al.*, 1997; Ellis *et al.*, 2000; Camper, 2004). Einsiedl *et al.* (2007) observed a complete molecular-level turnover of FAs and inferred that they must be perceived as a rather active participant in the global carbon cycle in a karst aquifer as a consequence of its high molecular-level alterations. Cunha-Santino and Bianchini Jr. (2008) suggested that in the Infernão lagoon humic substances mineralization is faster under low dissolved oxygen than under high dissolved oxygen conditions.

Measuring the contribution of different DOM constituents to the metabolism of heterotrophic bacteria has been hindered by methodological problems. The presence of poorly characterized organic complexes and numerous individual organic molecules at low concentrations makes comprehensive measures of uptake rates difficult on a molecular basis. Composite measurements, such as dissolved organic carbon (DOC) or humic substances analyses are typically not sensitive enough to detect significant uptake without extended periods of incubation, concentration of substrate (Meyer *et al.*, 1987), or both (Moran and Hodson, 1990, Moran and Hodson, 1994). Furthermore, chemical analysis does not determine what portion of the organic carbon is actually bioavailable. Bioavailable forms of organic matter make up only a small portion of the total organic matter in aquatic ecosystems (Münster, 1993). Methods for measuring labile matter are generally based on some consequence of microbial activity. As a result, the utilization of DOM components is often inferred from the growth of bacteria (Tranvik, 1988; Hopkinson *et al.*, 1998). One approach looks at the increase in bacterial biomass (traditionally total countable cells, TC, and culturable cells as colony forming units, CFU) as a consequence of growth. The enumeration of the total countable cells in water samples and in diluted biofilm suspensions is usually performed with a blue fluorescent nucleic acid stain, namely, DAPI (4',6-diamino-2-phenylindole). DAPI binds preferentially to double-stranded DNA forming a stable complex which fluoresces about 20 times more than unbounded. The heterotrophic plate count is a procedure for estimating the number of colony forming units

which corresponds to the culturable bacteria. The fraction of volatile suspended solids (VSS) is also considered as a measure of bacteria concentration (Tchobanoglous *et al.*, 2003). VSS are usually measured according to the gravimetric method described in standard methods (APHA *et al.*, 1995). Other useful method to estimate bacteria growth is the protein content (Zubkov *et al.*, 1999) which may be measured according to the Lowry method (Lowry *et al.*, 1951), modified by Peterson (1977).

1.4 BIOFILM

Many definitions of biofilm exist in the literature (Wimpenny, 2000). They all have in common that biofilms are described as populations of microorganisms of different types (bacteria, algae, protozoa and fungi) which accumulate at interfaces and excrete extracellular polymeric substances (EPS) that form a highly hydrated slime in which the cells are embedded (Hall-Stoodley *et al.*, 2004; Wimpenny, 2000; Branda *et al.*, 2005). EPS are composed of proteins, polysaccharides, lipids, ribonucleic acids and humic substances that are present sometimes in substantial amounts (Frolund *et al.*, 1996; Zhang *et al.*, 1999; Sutherland, 2001b; Flemming and Wingender, 2003). Their composition and amount are highly influenced by the type of microorganisms and environmental conditions such as nutrients, temperature, pH and hydrodynamics (Sutherland, 2001a; Melo, 2003). According to Characklis *et al.* (1990), bacteria are generally dominant in whatever biofilm due to the high growth rates, low sizes, adaptation capacity and extracellular polymers production.

In natural aquatic environments, conditions are often harsh with a constant competition for resources. Adherent microbial populations were early on observed to contain more bacterial cells than in the pelagic population (Zobell, 1943) and attachment to a surface could represent a survival strategy, with possibilities for higher flux of nutrients (Characklis, 1981; Kjelleberg, 1982; Marshall, 1988).

The major component of the biofilms is water, representing from 70 to 99 % (Watnick and Kolter, 2000; Flemming *et al.*, 2002; Stoodley *et al.*, 2002). In general, biofilms are viscoelastic and coloured white/yellow to brown/black. Since EPS have a mucilaginous consistency, the adsorption of particles and cells can occur, even for those that are not able to produce adhesion polymers.

1.4.1 Biofilm Formation

Biofilm development on a solid surface in contact with water is schematically represented in Figure 1.4 and an example of river biofilm is depicted in Figure 1.5.

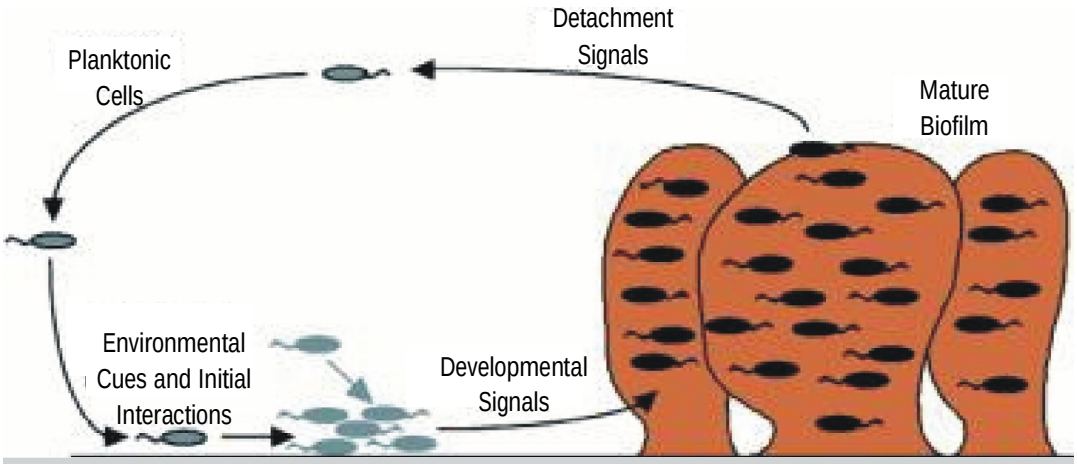

Figure 1.4 Schematic diagram of biofilm formation and growth. Adapted from O'Toole *et al.*, 2000.

Figure 1.5 Biofilms formed in the river Cávado (Braga, Portugal).

The formation of biofilms has been proposed to be a developmental process wherein planktonic (free-swimming) bacteria adapt to life on a surface (Davey and O'Toole, 2000). It is the result of several physical, chemical, and biological processes occurring simultaneously, including:

1) formation of a conditioning film;

2) attachment;

3) biofilm growth;

4) biofilm maturation – equilibrium between accumulation and detachment.

Conditioning film. Regardless of the environment, biofilm formation commences with the physical or chemical adsorption of a conditioning film, made up of polysaccharides, proteins, lipids, humic acids, nucleic acids and aromatic amino acids to which the early colonizing bacteria subsequently adhere (Bakker *et al.*, 2004; Bhosle *et al.*, 2005).

Attachment. Surfaces in aqueous environments that contain organic matter will be covered by an adsorbed layer of molecules, a conditioning film, which form before any microorganisms attach to the surface (Bos *et al.*, 1999; Tsibouklis *et al.*, 1999). Consequently, the strength of the attachment is to a large extent dependent on the cohesiveness of the conditioning film (Bos *et al.*, 1999).

For pelagic bacteria to encounter a surface, microbial transport could be either passive, by diffusion, fluid dynamic forces and sedimentation; or be due to active motility or chemotaxis (Bos *et al.*, 1999; Marshall, 1986). Since a bacterium is in the close neighbourhood of a surface it will be attracted to the surface by Lifshitz-van der Waals forces. Most surfaces in natural environments and most bacteria have a negative surface, therefore a layer of positively charged counter-ions will surround them. For attachment to occur, the attractive Lifshitz-van der Waals forces have to overcome the repulsive forces induced by the electrostatic interations. This interaction has been theoretically described by the Derjaguin, Landau, Verwey and Overbeek theory (DLVO theory) (Bos *et al.*, 1999; Hermansson, 1999). Hydrophobic interactions are also involved in initial attachment. Since two surfaces are in close contact, the structural ordering of water molecules between the surfaces result in hydration forces. The resulting force will be repulsive for hydrophilic surfaces and attractive for hydrophobic surfaces (Rijnaarts *et al.*, 1995). Another approach to view surface attachment is represented by the thermodynamic approach where attachment is thermodynamically favoured when the interfacial tension between the surface-bacterium is lesser than that of the surface-liquid and bacterium-liquid together (Absolom *et al.*, 1983).

It is important to consider that theories for attachment treat the microorganisms as colloidal particles with homogeneous surfaces. Indeed the bacterial surface is a highly heterogeneous and complex structure that could contain capsule material, flagella, curli, outer membrane proteins and fimbriae that are involved in bacterial attachment (Otto *et al.*, 2001). Therefore, structural and functional changes of the bacterial surface may be induced as the bacterium encounters a surface, which would affect attachment (Davey and O'Toole, 2000; Hong and Elimelech, 1997). Environmental parameters that could induce attachment include osmolarity, temperature, pH, iron and oxygen availability and starvation (O'Toole *et al.*, 2000; Prigent-Combaret *et al.*, 2001).

Biofilm growth. When the attached bacteria start to grow they form microcolonies, i.e. clusters of associated bacteria, excreting organic polymers and initiating the formation of the biofilm matrix. Exopolysaccharide synthesis has been shown to be important for the formation of microcolonies, (Allison and Sutherland, 1987; Watnick and Kolter, 1999). As biofilm thickness increases, transport of nutrients from the external liquid media to the inner layers of biofilm and transport of excreted metabolites in the opposite direction are important for the biofilm maintenance. Throughout this phase, bacteria detachment events occur although at a lower extent compared to the growth rate.

Biofilm maturation. Mature biofilm is composed of an organized consortia of microorganisms embedded in an organic matrix which protects the bacteria. In a biofilm several processes may occur at the same time: bacteria detachment into water, attachment of planktonic bacteria, growth and death. Nevertheless in a mature biofilm these processes are at equilibrium and the attached cells per unit surface area are constant with time though with periodic fluctuations. At this phase, the biofilm should attain the highest thickness that is basically dependent on the hydraulic conditions, the mass transport and the biofilm cohesion.

Hydraulic shear stress provoked by high flow velocities leads to detachment of bacteria and biofilm aggregates (sloughing), with higher detachment rates at increasing shear (Characklis *et al.*, 1990). Detachment is said to be due to at least four different mechanisms (Bryers, 1988): erosion (the continuous release of single cells or small clusters of cells), sloughing (the rapid detachment of large portions of the biofilm), abrasion (collision of solid particles with the biofilm), and predator grazing. Erosion and sloughing can result from biofilm-associated processes, such as enzyme production (Allison *et al.*, 1998; Boyd and Chakrabarty, 1994; Lee *et al.*, 1996), chemical signal production (Puskas *et al.*, 1997), cell-cycle-mediated events (Allison *et al.*, 1990; Gilbert *et al.*, 1989; Gilbert *et al.*, 1993), and global regulation (Jackson *et al.*, 2002) or from external factors such as shear forces (Gilbert *et al.*, 1993; Picioreanu *et al.*, 2001), abrasion (collision of solid particles with the biofilm), and predator grazing (Stewart, 1993).

1.4.2 Biofilm Structure

Structure is clearly a very important, almost crucial factor in how the activity of the biofilm bacteria will be expressed (de Beer *et al.*, 1994; Massol-Deya *et al.*, 1995). Two mainly different conceptual models dealing with the structure and function of biofilms can be found in the literature (Bishop, 1997). The first one, the continuum model, is a conventional diffusion-based concept with division of

the biofilm in specific compartments, the substratum, the biofilm (subdivided in a surface and a base film), the bulk liquid and a possible head space. However, observations in natural aquatic environments and technical water systems as well as laboratory studies on submerged biofilms indicate that biofilms frequently display structural heterogeneities to varying degrees. Thus, the microcluster model, a complex three-dimensional model, was established, describing mushroom-shaped biofilm clusters separated by channels and with streamers on their "mounds".

In contrast to planktonic microorganisms, biofilms secrete multiple EPS, which can make up between 50-90 % of the total organic matter of biofilms. EPS determine the structural and functional integrity of microbial biofilms, and contribute significantly to the organization of the biofilm community (Branda *et al.*, 2005). EPS are involved in the formation and maintenance of a three-dimensional, gel-like, highly hydrated and locally charged (often anionic) biofilm matrix, in which the microorganisms are more or less immobilized. The biofilm matrix may trap inorganic particles and biogenic material, it may incorporate multivalent cations which can be involved in the cross-linking of anionic EPS and thus in polymer network formation.

Biofilm structure, stability, and cohesiveness is very complex and depends on many factors such as physical (porosity, density, EPS content) and mechanical (viscoelasticity) properties of the biofilm (Körstgens *et al.*, 2001; Möhle *et al.*, 2007; Stoodley *et al.*, 1999; Towler *et al.*, 2003). Additional factors that influence biofilm structure are the microbial composition, physiological response including growth yield, the nutrient availability, hydrodynamic conditions, temperature, pH and detachment (Ollos *et al.*, 2003; Lyautey *et al.*, 2005a).

Investigation of biofilm structure is however extremely difficult since most methods are invasive and disruptive (Characklis, 1981; Peyton and Characklis, 1995; Stoodley *et al.*, 1998; Stoodley *et al.*, 1999). In multispecies biofilms, cells grow in microcolonies within stable matrices. Unlike planktonic cells, biofilm bacteria live in microbial communities where the availability of nutrients and diffusion of metabolic products to one cell depends on the activity of neighbouring cells. Biofilm bacteria live in matrix-enclosed environments and although a mature biofilm is characterised by complex architecture around water channels, they are subjected to conditions of limited diffusion where matrices function as a barrier minimising the loss of nutrients (Lünsdorf *et al.*, 2000). In this way biofilm bacteria have an entirely different physiology from their planktonic counterparts.

Building evidence (Jackson *et al.*, 2001; Lyautey *et al.*, 2005b; Martiny *et al.*, 2005) suggests that initial community formation is stochastic and largely driven by the recruitment of species from the bulk liquid. As microbial cells become more abundant with biofilm growth, competition for resources may become increasingly important and less-competitive microorganisms are out-competed.

Diversity would decrease during this developmental phase, with a few competitors becoming predominant. Finally, as biofilms mature, the community can become more diverse through niche diversification and internal recycling of resources. Differential aggregation behavior of bacteria may also lead to higher diversity in biofilms growing under low flow than in biofilms growing under high flow (Rickard *et al.*, 2004). Despite these various pieces of evidence, there is still lack a mechanistic understanding of how physical and biotic controls may collectively affect biofilm formation and succession. Recently, Battin *et al.* (2007) suggested viewing biofilms as microbial landscapes to clearly link the hydrodynamics of the bulk liquid, the biofilm topography (i.e., the landscape), and the immigration rate of cells. Their framework thus recognizes the function of coupled physical processes (e.g., hydrodynamics and topography) and demographic biological processes that are largely stochastic in nature (e.g., death and reproduction) in the assembly of biofilm communities.

River biofilms produced different amounts of polysaccharides when nutrients availability and flow velocity changed (Staudt *et al.*, 2004). It is commonly agreed that flow velocity represents an important factor for the structure and dynamics of aquatic ecosystems, especially in running waters and tidal regions (Battin *et al.* 2003a, b). Flowing water enhances the exchange of nutrients and gases such as oxygen between the water and biofilm communities affecting their metabolic activity (Silvester and Sleigh 1985; Stevens and Hurd 1997). Recently, Besemer *et al.* (2007) verified that flow velocity clearly shaped the development of biofilm architecture and community composition. Since rivers have a wide range of flow velocities its effect on structure-function coupling of biofilms is largely studied in laboratory-scale reactors. 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; Wäsche *et al.*, 2004; Garny *et al.*, 2008). Simões *et al.* (2006), reported higher bacterial numbers in biofilms at turbulent conditions (Re = 11000) than in the laminar flow (Re = 2000), the carbon concentration in both cases being 0.5 mg/L. Other studies are in agreement (Percival *et al.*, 1999; Lehtola *et al.*, 2006; Paris *et al.*, 2007), especially if the amount of biodegradable matter is low (0.5 mg/L) (Ollos *et al.*, 2003). This is attributable to better mass-transfer of growth limiting nutrients at the higher flow velocity of water.

Manuel (2008), however, obtained contradictory results in the study of biofilm formation with a constant carbon concentration at laminar regime (Re = 1001) and turbulent regimes (Re = 5797 and Re = 8293) and observed that the total countable cells (TC) decreased with the Reynolds number increase, similar trend of results were obtained by Donlan and Pipes 1988; Soini *et al.*,

2002; Ragazzo, 2002; Stoodley *et al.*, 2002; Chen *et al.*, 2005 and Tsai, 2005. In fact, increase of flow velocities enhances biofilm detachment.

Significant changes in microbial community structure have also been observed in response to nutrients; however, limited data exist on their impact on the chemical nature of EPS in river biofilm systems (Neu *et al.*, 2005). In these natural systems, the heterotrophic biofilm microorganisms commonly satisfy their energy and carbon demand from dissolved organic carbon (DOC) that is immobilized from the pore water by the EPS matrix. DOC chemical composition largely reflects catchment and channel processes that, along with flow paths and seasonality, determine the contribution of DOC from different sources (allochthonous vs autochthonous). Terrigenous DOC enters streams predominantly during storms (e.g. Kaplan and Newbold, 1993) and is often rich in humic substances and their precursors. Freeman and Lock (1992) showed that high molecular weight fractions of DOC can inhibit biofilm metabolism in brown-water rivers. Three years after, Freeman and Lock (1995) described the biofilm matrix as a storage site for organic substrates that prevents heterotrophic bacteria from starvation during times of low carbon supply. More recent studies pointed out that DOC might form complexes with enzymes present in the biofilm matrix reducing its hydrolytic activity (Boavida and Wetzel, 2002; McNamara and Leff, 2004).

Temperature and pH represent also two important factors that affect life by modifying the electrostatic interactions between surfaces and micoorganisms, enzymatic activity, kinetics and equilibrium of reactions, and many other properties. Particularly, in river biofilms, pH levels are generally close to neutrality and temperatures may vary from 0.9 to 31.6 °C (Webb and Walling, 1992; Markich and Brown, 1998).

1.4.3 Biofilm Metabolism

Dissolved organic carbon (DOC) is removed from river water by both abiotic and biotic processes (Allan, 1995) and the main biotic processes are uptake by microorganisms, principally bacteria. Regarding this uptake, biofilms play an important role in the retention and storage of DOC (Marmonier *et al.*, 1997; Battin *et al.*, 1999), and may contribute to the self-purification capacity of streams and rivers (Pusch *et al.*, 1998; Sabater *et al.*, 2002). The metabolic activity of biofilm bacteria may be influenced by the ambient concentration and composition of DOC (Fischer *et al.*, 2002). However, biofilms can buffer the supply of organic substrates so that short-term changes in the quality and quantity of DOC do not necessarily exert an immediate effect on their metabolism (Freeman and Lock, 1995). Uptake of DOC can be directly diffused via membrane permeases or via

diffusion through pores if the molecules are small enough (Nikaido and Vaara, 1985). In the case of large molecules bacteria can produce extracellular enzymes required to its degradability and effectively use (Fischer, 2003). High molecular weight compounds (>10.000 Da), comprising macromolecules such as humic substances, proteins, polysaccharides and lipids complexes, forms the major part of the organic matter in aquatic environments (Cole *et al.*, 1984; Lock, 1990; Klavins, 1998). Otherwise, DOC of low molecular weight, such as monosaccharides and certain labile sources, are taken up most quickly by heterotrophic bacteria (Kapplan and Bott, 1982; Meyer *et al.*, 1987; Kaplan and Bott, 1989). Extracellular enzymes are essential to initiate the remineralization of high molecular weight organic matter, since heterotrophic bacteria can not take them directly from the surrounding medium into the cell and, therefore, they need to be hydrolysed outside the cell to sizes small enough to permit transport across the outer membrane (Arnosti and Jørgensen, 2003). Thus, extracellular enzymatic approach provides powerful information for understanding crucial processes of decomposition and microbial activity in streamwater ecosystems (Chróst, 1990; Lock, 1990). Extracellular enzymes are either attached to the outer membrane or released, free, into the surrounding solution (Chróst, 1991). Their production and activity are in general closely controlled by a microbial cell, due the fact that represents an investment of energy, and consequently, continuous synthesis in the absence of substrates is unnecessary (Chróst, 1991). Some enzymes are expressed constitutively, but many more are induced under specific circumstances (Chróst, 1991; Arnosti and Jørgensen, 2003). Extracellular enzyme production may be further regulated by catabolite repression, in which the presence of a substrate (usually an easily metabolised carbon source) may prevent production of enzymes required for metabolism of a more complex substrate (Chróst, 1991; Priest, 1992). Generally, hydrolytic enzymes can be classified based on the type of reaction catalysed.

1.5 BIOFILMS IN NATURAL SYSTEMS

Rivers are one of the main sources of drinking water (Pimentel *et al.*, 1997) and the requisite to provide drinking water of high quality is continuously confronted, since river water has to be treated by progressively more sophisticated and costly procedures to achieve full purification. The study of natural processes that occur in flowing waters, affecting the water quality, can provide a cost-effective amelioration treatment. This is the case of dissolved organic carbon (DOC) dynamics in natural water systems. The presence of high levels of DOC in surface waters represent a problem for water industry due the fact that they are associated with the formation of carcinogenic disinfection by-products, namely, trihalomethanes (THM), upon chlorination of drinking water. So, THM presence in drinking water has given rise to health concerns (Bull *et al.*, 1995; Nieuwenhuijsen *et al.*, 2000). One of the methods of minimizing their formation would be the reduction or removal of DOC prior to chlorination. Therefore, a better understanding of the biofilms involvement in DOC consume, could lead to a biological pre-treatment application in drinking water plants. In fact, biofilms represent the dominant microbial community form in all aquatic ecosystems and are responsible for providing a wide variety of ecosystem functions (Costerton *et al.*, 1995; Watnick and Kolter, 2000; Battin *et al.*, 2003b; Battin *et al.*, 2007).

Biofilms are now recognized as complex and dynamic communities in which substantial phenotypic diversification allow microorganisms to adapt to different environment conditions (Battin *et al.*, 2008). As biofilms grow and thicken form physical and chemical gradients, producing niches for a wide variety of lifestyles. They are also capable of actively releasing plaktonic cells into the surrounding aquatic environment, allowing microbes to colonize new or more favorable niches (Watnick and Kolter, 2000; Webb *et al.*, 2003; Hall-Stoodley *et al.*, 2004; Parsek and Fuqua, 2004). They can sorb water, inorganic and organic solutes and particles. EPS, cell walls, cell membranes and cell cytoplasm can serve as sorption sites. These sites display different sorption properties, preferences and, capacities (Späth *et al.*, 1998). The heterotrophic biofilm microorganisms commonly satisfy their energy and carbon demand from DOC that is immobilized from the porewater by the EPS matrix.

DOC, inorganic nutrients as well as flow velocity have been proved to influence microbial abundance in aquatic biofilms (Kaplan and Bott, 1989; Leff and McArthur, 1990; Rier and Stevenson, 2001; Rier and Stevenson, 2002; Kirchman, 1994; Battin, 2003a; Lawrence and Neu, 2003; O'Sullivan *et al.*, 2002; Sobczak, 1996; Tank and Webster, 1998). The source, quality and type of DOC, as well as, the quantity, may influence the abundance and distribution of bacteria in stream ecosystems

(Koetsier *et al.*, 1997; Leff, 2000; Leff and Meyer, 1991). In freshwater systems, the major bacterial groups are the alpha, beta, gamma-Proteobacteria and the Bacteroidetes (Manz *et al.*, 1999; Araya *et al.*, 2003; Gao *et al.*, 2005; Besemer *et al.*, 2007). These groups included bacteria that are important to the cycling of carbon and nutrients. The alpha-Proteobacteria, in general, dominate natural freshwater systems that are oligo- or mesotrophic (Pinhassi and Berman, 2003; Olapade and Leff, 2005). This bacterial group may also have a preference for labile organic matter (Zwisler *et al.*, 2003). The beta-Proteobacteria group contains the vast majority of freshwater ammonia-oxidizing bacteria (Purkhold *et al.*, 2003). The beta-Proteobacteria is also considered a diverse and opportunistic group that is found in large quantity in a variety of conditions, including polluted rivers (Brümmer *et al.*, 2003; Manz *et al.*, 1999). The gamma-Proteobacteria may be an opportunistic group under high-nutrient conditions, presenting a lower activity in oligotrophic systems (Pinhassi and Berman, 2003). They also contain some of the common bacteria which are known as pollutant degraders (Uta *et al.*, 2005) and some of the main aquatic denitrifiers (Gómez-Villalba *et al.*, 2006). Bacteroidetes group is known for using high molecular weight carbon compounds including cellulose and chitin (Kirchman, 2002) and being highly abundant during the winter months associated to low temperatures (Araya *et al.*, 2003).

The compositions of bacterial communities in streams have been studied using classical methods based on cultivation techniques (Baker and Farr, 1977; Gray, 1951; Leff, 2000). Reasoner (2004) stated that the use of low nutrient media (R2A) to cultivate cells during an extended incubation time (seven days) favor the growth of slow-growing and indigenous aquatic bacteria. Defives *et al.* (1999) reached 65 % of culturability in a French mineral water using R2A, and Foght *et al.* (2004), using the same media, achieved values of 82 % of culturability in a subglacial sediment. However, there are authors that obtained different results and pointed out that cultivation techniques suffer from a number of artifacts, mainly because only a small, and probably not representative, number of bacteria are cultivable and identifiable (Amann *et al.*, 1995). Culture based methods greatly underestimate the diversity of natural microbial communities and may select for organisms which either do not make up a significant portion of the community or do not contribute a great deal to the activity of the community, providing a skewed representation of what is in fact present.

Rapid advances in molecular and biochemical methods have allowed microbial communities to be described without the bias involved in culturing techniques (Amann and Kühl, 1998). They have also become increasingly sensitive, enhancing the ability for measurement on finer and finer scales. The small subunit ribosomal RNA (SSU rRNA) sequence information, also known as 16S rRNA for prokaryotes, has been used to study microbial communities. Nowadays, the 16S rRNA gene is still

the most widely used molecular phylogenetic marker in microbial ecology studies. The application of the polymerase chain reaction (PCR) has been the most significant development for dealing with environmental microorganisms, allowing replication of target rRNA genes in order to make them detectable (Giovannoni, 1991). Cloning and sequencing of 16S rRNA genes provide information about the genetic diversity and phylogenetic relationships between microorganisms present in a specific ecosystem, after comparative analysis of the retrieve 16S rRNA gene sequences with 16S rRNA gene databases (Amann *et al.*, 1995; Godon *et al.*, 1997).

Fingerprinting methods such as denaturing or temperature gradient gel electrophoresis, DGGE or TGGE respectively, are considered suitable tools for rapid and comparative analysis of unknown natural communities (Ranjard *et al.*, 2000). DGGE is based on electrophoresis of PCR products (rRNA amplicons) in polyacrylamide gels containing a linearly increasing gradient of DNA denaturants (generally urea or formamide) (Muyzer, 1999). In DGGE, DNA fragments of the same length, but with different base-pair sequences, can be separated for PCR products not longer than 500 bp. This analysis has been recently used for studying stream biofilms with the advantage to get an overall impression of the heterogeneity of the microbial community as well as to quantify individual differences and temporal changes in the predominant community (Jackson *et al.*, 2001; Araya *et al.*, 2003; Lyautey *et al.*, 2003; Lyautey *et al.*, 2005a; Besemer *et al.*, 2007). However, it has disadvantages as well; one of the most important is that it cannot readily be used to determine the abundance of particular taxa because of biases in PCR (Hansen *et al.*, 1998; Becker *et al.*, 2000). Thus, it generally provides presence/absence data rather than information relative to actual abundances.

The abundance of biofilm bacteria can be monitored using methods such as total countable cells, TC, culturable cells (colony forming units, CFU), volatile suspended solids (VSS) and protein content as previously described in the part 1.3.4.

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2 | QUANTIFICATION OF HUMIC ACIDS IN SURFACE WATER: EFFECTS OF DIVALENT CATIONS, pH AND FILTRATION

Humic acids (HAs) content of raw water is an important analytical parameter in water treatment facilities because HAs in the presence of chlorine may lead to the formation of dangerous by-products (e.g., trihalomethanes). The concentration of HAs in water is not directly accessible by common analytical methods due to their heterogeneous chemical structure. The aim of this study was to compare two methods to assess humic acids (HAs) in surface water namely absorbance of ultraviolet light at 254 nm (UV_{254}) and total organic carbon (TOC), as well as to evaluate the effects of calcium and magnesium concentrations, pH and sample filtration on the methods' results. An aqueous solution of a commercial HA with 10 mg/L was used in the present work. Quantification of the HA was carried out by both UV_{254} and TOC (combustion-infrared method) measurements. UV_{254} results were converted to TOC using a calibration curve. The effects of calcium (0 - 136.3 mg/L) and magnesium (0 - 34.5 mg/L) concentrations, pH (4.0, 7.0 and 9.0) and sample filtration on UV_{254} and TOC measurements of the HA suspension were evaluated. More accurate TOC values of HA suspensions were obtained by the combustion-infrared method than by the UV_{254} absorbance method. The higher differences of TOC values between unfiltered and filtered samples were detected in the presence of calcium at pH 9.0 using the spectrophotometric method.

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2.1 INTRODUCTION

Natural organic matter (NOM) concentration in surface water typically range from 0.1 mg/L to 20 mg/L and is mainly composed of humic substances (HSs) (Volk, 2001). HSs composition varies from source to source as they result from microbiological, chemical and photochemical transformations of plant and animal residues (Rose and Waite, 2003). The main constituents of HSs include aromatic and aliphatic structures as well as carboxylic, phenolic-OH, amino and quinone groups. These groups may be evaluated by nuclear magnetic resonance (NMR) spectroscopy which is by far the single most powerful tool for structural HSs studies (Simpson, 2002). The fraction of HSs that is soluble at high pH but insoluble under acid conditions is denominated as humic acids (HAs) (Claret *et al.*, 2003). In general, HAs present in surface water carry an electric negative charge (zeta potential), associated to the dissociation of carboxylic and phenolic-OH groups that can be determined by electrophoretic mobility (Weber, 1988; Warwick *et al.*, 2001). In the presence of divalent cations (e.g. calcium), HAs form intramolecular and intermolecular aggregates due to charge neutralization and functional group bridging (Wandruszka *et al.*, 1997; Anđelković *et al.*, 2004).

The HSs content of raw water is an important analytical parameter in water treatment facilities since they have been associated with the formation of carcinogenic disinfection by-products (e.g. trihalomethanes) upon chlorination of drinking water (Wang and Hsieh, 2001). The concentration of HAs in water is not directly accessible to common analytical methods due to their heterogeneous chemical structure (Vogel *et al.*, 1999; Mertig *et al.*, 2002). Instead, total organic carbon (TOC) and absorbance of ultraviolet light at 254 nm (UV_{254}) are used as group parameters to represent the concentration of HSs in water (Najm *et al.*, 1994; Eaton, 1995). Spectrophotometry utilizes the capacity of HSs to absorb ultraviolet light at 254 nm, which increases with their content of aromatic rings, the ratio between aromatic and aliphatic carbon, the total carbon content in the water, and the molecular weights of the HSs (Vogel *et al.*, 1999). Given stable absorption characteristics, the UV light absorbance of HSs at a specific wavelength is proportional to their concentration according to Beer's law. TOC content has been widely used as a standard method for monitoring NOM in surface waters (Tipping *et al.*, 1997). This method is able to monitor organic carbon independently of their molecular configuration; therefore the chemical reaction between constituents has less influence in this results accuracy than the same results obtained by spectrophotometric method. Comparing their practical application, spectrophotometric method requires less expensive equipment than TOC

method (sample combustion followed by infrared CO_2 detection). It is also easier to maintain and operate, as the only sample pretreatment necessary is filtration and the time necessary to analyze each sample is approximately one minute (Skoog and Leary, 1988). The main disadvantage of spectrophotometry, however, is its possible corruption by chemical reaction between constituents of the solution, namely molecular aggregations.

Although several investigations have reported either on the quantification of HSs using ultraviolet UV and TOC methods or on the aggregation of HSs under certain environmental conditions, little attention has been given to the effect of divalent cations (namely calcium and magnesium), sample pre-treatment and pH on methods' results (Wang *et al.*, 2001; Baker, 2002; Volk *et al.*, 2002; Gan *et al.*, 2007; Benedetti *et al.*, 1996; Pandey *et al.*, 2000; Koopal *et al.*, 2001; Majzik and Tombácz, 2007). The aim of this study was to compare two methods to assess humic acids (HAs) in surface water namely absorbance of ultraviolet light at 254 nm (UV_{254}) and total organic carbon (combustion-infrared method), as well as to evaluate the effects of calcium and magnesium concentrations, pH and sample filtration on the methods' results.

2.2 MATERIALS AND METHODS

2.2.1 Humic acid characterization

A commercial humic acid (HA) from Fluka (commercialized by Sigma Aldrich, 53680 Humic acid) was used to simulate humic substances (HSs) in surface water. Its elemental composition was determined with an elemental analyzer (Carlo Erba, model EA 1108). Functional groups were identified by ^{13}C solid-state and ^{1}H solution nuclear magnetic resonance (NMR). ^{13}C NMR was carried out using a Brucker MSL 400P operated at ^{13}C frequency of 100.63 MHz and magic-angle spinning rate (MAS) of 6.0 kHz. The solid HA samples were filled into a 4-mm diameter ZrO_2 rotor with a Kel-F cap (1.2 s recycle time and 1 ms contact time). Each spectrum consisted of 2400 data points and the chemical shifts were referenced externally to glycine (176.03 ppm). ^{1}H-NMR was carried out using a Varian Unity Plus 300 spectrometer NMR with a 5 mm probe head. The solid HA, 20 mg, was dissolved in 1 mL of dimethylsulfoxide (DMSO-d_6) and deuterated sodium hydroxide solution ($NaOD/D_2O$) and was used for standard measurements (45° pulse acquisition, 1 s delay).

2.2.2 Experimental design

Total organic carbon concentration of HA solutions (10 mg/L of C) in the presence of different concentrations of calcium (0 - 136.3 mg/L) and magnesium (0 - 34.5 mg/L) ions, at three pH values (4.0, 7.0 and 9.0) was directly measured by the combustion-infrared-method and indirectly determined by UV spectroscopy at 254 nm. Unfiltered and filtered samples were analyzed. Filtered samples were obtained using a 0.45 mm filter (514 - 4156 Membrane disc filters Supor-450, VWR). Experiments were performed in triplicate.

2.2.3 Humic acid solutions

A concentrated stock solution was prepared by dissolving 206.8 mg of humic acid (HA) in 1 L of an aqueous solution of 4 g/L of NaOH. The stock solution was stored in the dark at 4 ºC. A series of solutions were prepared by dissolving a weighed amount of $CaCl_2.2H_2O$ or $MgSO_4.7H_2O$ (Sigma Aldrich, p.a.) in a ten-fold dilution of the HA stock solution (20.68 mg/L final concentration or 10 mg/L carbon). Experiments were performed at three different pH values (4.0, 7.0 and 9.0) which were adjusted by addition of HCl (73.0 mg/L) or NaOH (80.0 g/L) concentrated solutions. Ultrapure water was used in the preparation of all solutions.

2.2.4 Total organic carbon

Total organic carbon was determined by sample combustion and infrared carbon dioxide detection (5310 B) according to Standard Methods using a Shimadzu TOC-5000A analyzer (APHA, 1995).

2.2.5 UV spectroscopy

UV absorbance of humic acid solutions was carried out at 254 nm using a single beam spectrophotometer UV-160A (model STR-458) and a 1 cm quartz cell and the respective values were converted to total organic carbon using a calibration curve. For that purpose, serial dilutions of the concentrated HA stock solution in the range 0 - 41.4 mg/L (or 0 - 20 mg/L of carbon, based on HA elemental composition) were prepared at three pH values namely 4.0, 7.0 and 9.0. pH adjustment was achieved as previously described. The mathematical relationship absorbance versus concentration is presented in the Table 2.1.

Table 2.1 Linear regression equations from standard calibration.

		Sample	
pH		**Filtered**	**Unfiltered**
4.0	Equation	$A = 4.69 \times 10^{-2}$ TOC + 5.10×10^{-3}	$A = 6.06 \times 10^{-2}$ TOC - 4.00×10^{-3}
	R^2	0.9995	0.9999
7.0	Equation	$A = 5.48 \times 10^{-2}$ TOC - 3.60×10^{-3}	$A = 6.33 \times 10^{-2}$ TOC - 4.40×10^{-3}
	R^2	0.9998	0.9999
9.0	Equation	$A = 5.64 \times 10^{-2}$ TOC + 1.20×10^{-3}	$A = 6.43 \times 10^{-2}$ TOC + 1.00×10^{-7}
	R^2	0.9999	1

A: absorbance; TOC: total organic carbon in mg/L

2.2.6 Zeta potential of HA in the presence of divalent cations

The colloid's zeta potential of HA in the presence of calcium and magnesium was determined using a Malvern Zetasizer instrument. The zeta potential cell (DTS1060) was rinsed using a disposable syringe (DTS1060) with at least 20 mL of each sample solution before measuring the zeta potential of the HA in the test solution. All experiments were carried out at 20 °C using suspensions aged for 24 h. The zeta potential was derived from the electrophoretic mobility using the Smoluchowski approximation (Hunter, 1981).

2.2.7 Statistical analyses

A *t*-test was used where specific means were being compared. Acceptance or rejection of the null hypothesis was based on a α-level of 0.05 in all cases (Miller and Miller, 1992).

2.3 RESULTS AND DISCUSSION

2.3.1 Humic acid characterization

The elemental composition of the Fluka humic acid (HA) determined in the present study was 48.36 % of C, 26.91 % of O, 4.24 % of H, 0.78 % of N and 0.78 % of S. This result is in agreement with the one provided by Fluka (47.9 % of C, 4.91 % of H, 0.67 % of N and 1.18 % of S) but both differ from the one published by Pluciński *et al.* (54.37 % of C, 39.84 % of O, 4.28 % of H, 0.66 % of N and 0.85 % of S) (Pluciński *et al.*, 2007).

To obtain additional information regarding the structure of the HA, the atomic H/C, N/C, and O/C ratios has been determined. Previous studies suggest that the H/C ratio is an indicator of the amount of saturation of C atoms and/or branched structures within the molecule, the N/C ratio is related to the amount of proteinaceous compounds, and the O/C ratio is assumed to indicate the carbohydrate content and degree of oxidation (Giovanela *et al.*, 2004; Abbt-Braun *et al.*, 2004; Santos *et al.*, 2007). Literature studies reported H/C ratio for soil HAs in the range of 0.78 - 1.60 and for aquatic HAs between 1.23 - 1.46 (Geyer, 1998; Li *et al.*, 2003; Giovanela *et al.*, 2004). Regarding the O/C ratio, values reported for soil HAs are in the range of 0.35 - 0.59 while for aquatic HAs the values are between 0.55 - 0.63, respectively. The N/C ratio presented values ranging from 0.021 to 0.080 for soil HAs and from 0.036 to 0.070 for aquatic HAs. The atomic ratios determined in the present study were 1.04 (H/C), 0.42 (O/C), and 0.012 (N/C) which are within the values reported in the literature for soil HAs, with the exception of the N/C ratio. This value is below the lower limit reported, and probably reflects a chemical composition with a poor content of aminoacid units. Additional information on the structure of Fluka HA (Sigma-Aldrich) was obtained from FTIR and NMR results.

^{13}C Solid-state NMR and ^{1}H solution NMR spectra are depicted in Figure 2.1 and Figure 2.2, respectively.

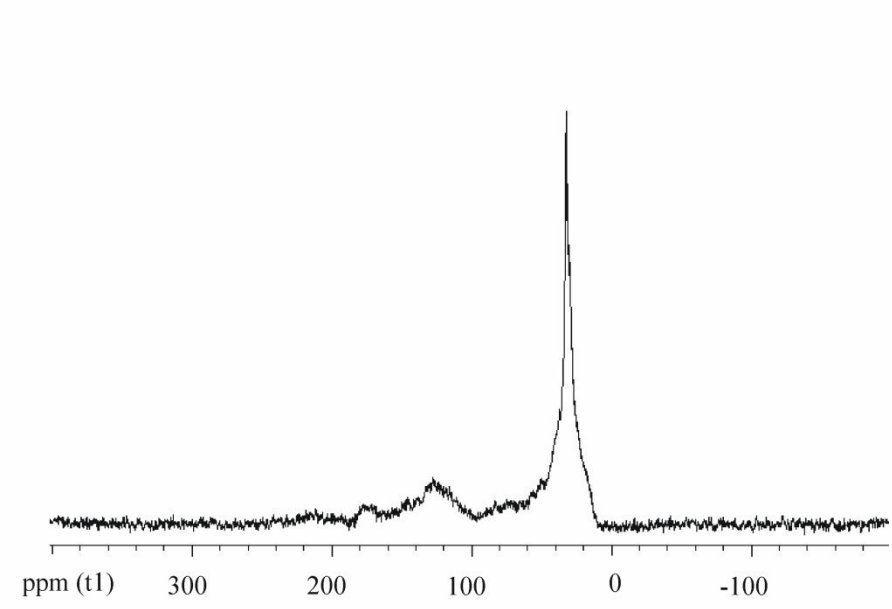

Figure 2.1 Solid-state ^{13}C NMR (^{13}C-NMR-MAS) spectrum of the HA.

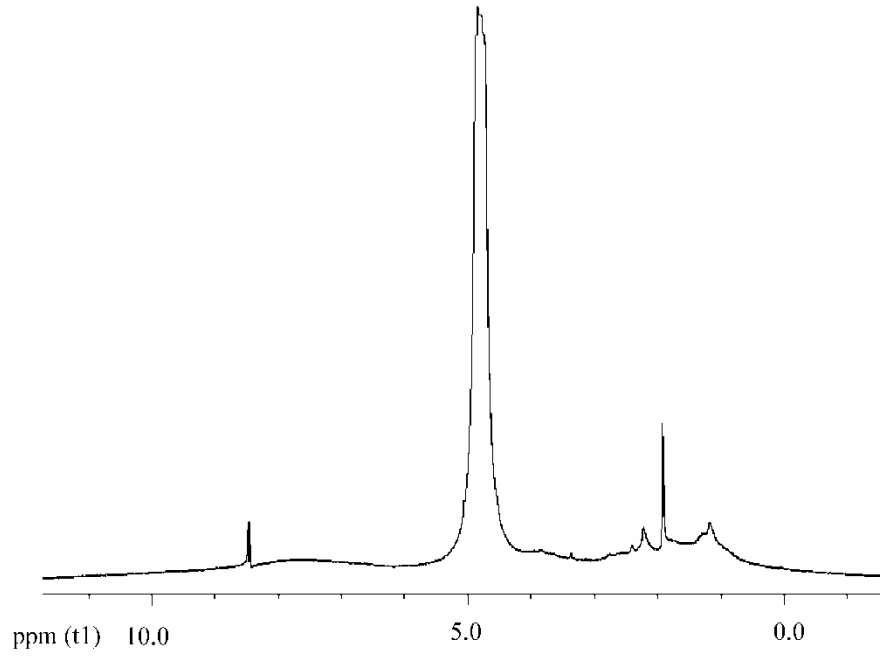

Figure 2.2 Solution H^1 NMR spectrum of the HA.

The ^{13}C NMR spectrum shows broad signals between δ 10 ppm and 225 ppm from which a sharp and intense singlet emerges at δ≈30 ppm. This signal must be associated with an equally intense singlet in the ^{1}H NMR spectrum of the same sample at δ 1.93 ppm, typical of acetate methyl groups. Other sharp but less intense signals at δ 1.19 ppm and δ 2.23 ppm in the ^{1}H NMR spectrum may indicate that other methyl groups are present either as part of alkyl chains or linked to carbonyl, or to aromatic groups. They may be associated with two shoulders visible in the ^{13}C NMR spectrum around δ 25 ppm and 35 ppm. The remaining signals in the ^{13}C NMR also indicate the presence of non-polar aliphatic carbon atoms (δ 10-40 ppm, under the intense acetyl absorption) and O-alkyl/N-alkyl carbons (δ 40-90 ppm). A broad signal in the ^{13}C NMR spectrum between δ 100-160 ppm indicates the presence of aromatic groups with both electron-donating (amino/alkoxy/hydroxy substituents) and electron withdrawing groups (carboxylic acids and derivatives). The presence of carbonyl groups was confirmed by the broad signal between δ 170-225 ppm. A similar pattern was reported in the literature for the ^{13}C NMR spectrum of a solid purified Fluka HA sample (Pertusatti and Prado, 2007). In this case, five broad peaks were identified in the 0-50, 50-85, 85-105, 105-160 and 160-200 ppm regions, confirming the presence of the same type of functional groups. A notorious difference observed in the present study is the sharp and intense singlet at δ≈30 ppm, assigned to the acetate methyl group. As a result, comparatively small signals are registered for the remaining regions. Broadening of this band as it reaches the base line leads to

partial overlapping with the 85-105 ppm band identified in the literature and assigned to C-O in carbohydrate compounds.

The signals in the ^{1}H NMR spectrum are spread between δ 0.5-10 ppm and are, in general, very broad. The intense band centered at δ 4.8 ppm was assigned to the water peak due to the presence of a large amount of water. In this case, hydration molecules were reinforced by water molecules formed by deuterium exchange of all the hydroxy/amino protons (of alcohols, phenols, carboxylic acids and amines) present in HA with D_2O used as co-solvent. This allowed us to calculate the molar ratio of protons on saturated and unsaturated carbon atoms (approximately 2.1:1), from the integration of the signals in the δ 0.5-4.2 ppm and of the δ 5.6-10 ppm regions respectively.

The FTIR spectrum (Figure 2.3) shows an intense signal centered at ν 3429 cm^{-1} assigned to the N-H/O-H stretching vibrations, confirming the presence of alcohols/phenols, amines/amides and possibly carboxylic acids. Two medium intensity peaks at ν 2922 and 2852 cm^{-1} may be due to the C-H stretching vibration of alkyl chains. The stretching vibration of the aromatic C-H bonds, usually visible in the ν 3200-3000 cm^{-1} region, may be masked by the broad N-H/O-H signal. An intense band at ν 1608 cm^{-1} can be assigned to the C=O stretching vibration in the carboxylate function and also to the C=C stretching vibration in the aromatic ring and alkene groups. A shoulder around ν 1700 cm^{-1}, typical of the stretching vibration of carbonyl groups of ketones (including quinones) carboxylic acids and/or amides, confirms that these units are present in humic acid. The bending vibrations of methyl and methylene groups (ν 1387 cm^{-1}) and the stretching vibration of the C-O bond in alcohols, phenols and ethers (overlapped bands between ν 1000 and 1200 cm^{-1}) also support the presence of these functional groups. The main absorbance bands and the corresponding assignments are in agreement with data reported in literature (Pertusatti and Prado, 2007; Klavins and Eglite, 2002; Prado *et al.*, 2006; Prado and Airoldi, 2003; Ussirini and Johnson, 2003; Palladino *et al.*, 2007; Stevenson, 1982; Landgraf *et al.*, 1998; Kucerik *et al.*, 2004).

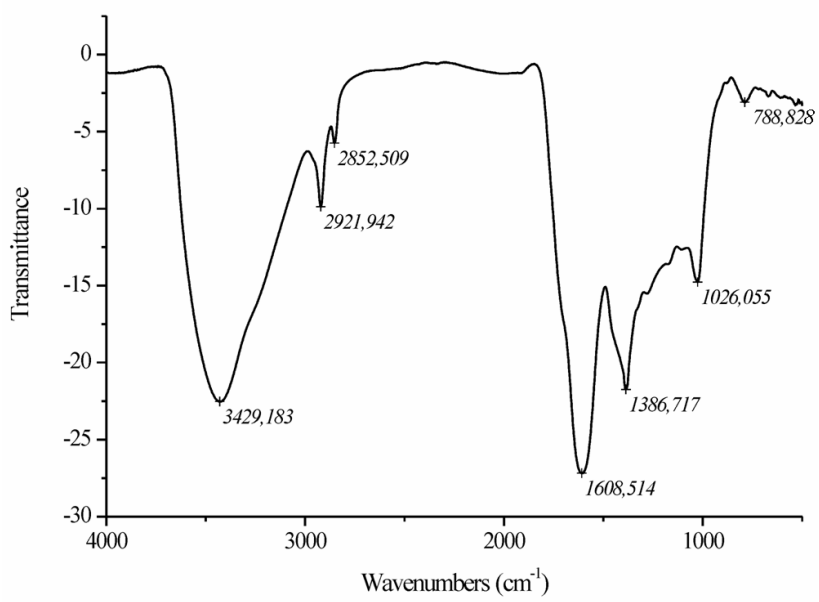

Figure 2.3 FTIR spectrum of the HA.

2.3.2 Zeta potential as a function of pH and concentration of divalent cations

Zeta potential (ZP) values became more negative, decreased from -38.4 ± 0.35 mV to -43.6 ± 0.30 mV, as pH was increased from 4.0 to 9.0. The variation of the colloid's zeta potential with pH reflects the ionization of the carboxylic and phenolic acidic groups (Alvarez-Puebla and Garrido, 2005). Charge as well as intra- and intermolecular electrostatic repulsion increase as acidic groups are ionized with increasing pH, restricting aggregation phenomena. The effect of the concentration of divalent cations on ZP values at different pH values is depicted in Figure 2.4 for calcium and in Figure 2.5 for magnesium.

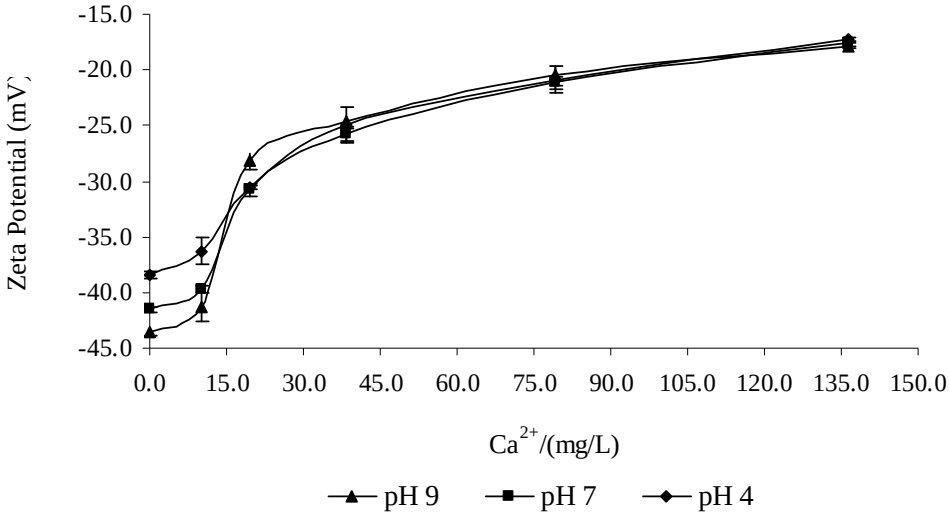

Figure 2.4 Zeta potential of the HA suspension (10 mg/L of carbon) as a function of pH at several calcium concentrations.

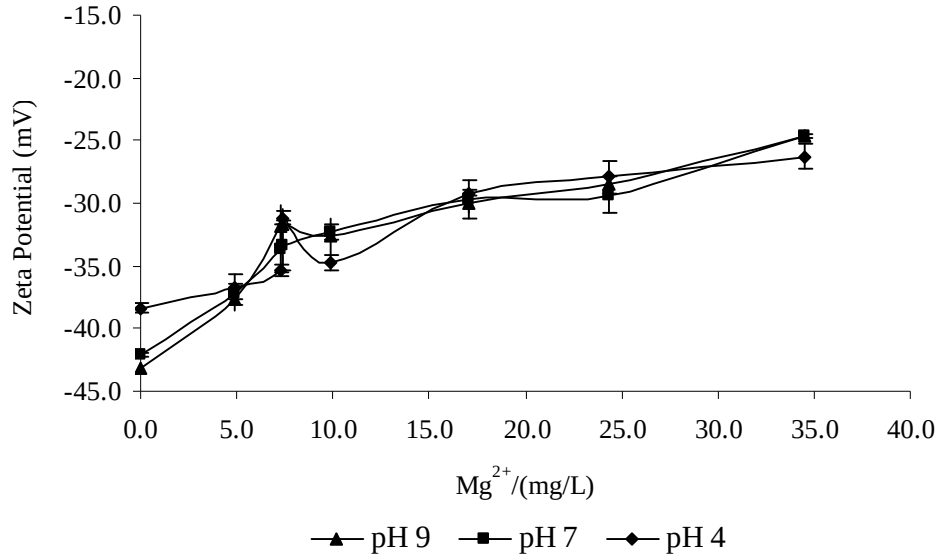

Figure 2.5 Zeta potential of the HA suspension (10 mg/L of carbon) as a function of pH at several magnesium concentrations.

The results show that ZP values increase (become less negative) with increasing calcium and magnesium concentrations, which can be explained by HA's charge reduction due to metal ions binding to the negatively charged carboxylic groups (Gregory and Duan, 2001; Schäfer *et al.*, 2002; Majzik and Tombácz, 2007). The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. A dividing line between stable and unstable aqueous dispersions is

generally taken at either +20 or -20 mV. Colloids with ZP more positive than +20 mV or more negative than -20 mV are normally considered stable (Elfarassi *et al.*, 1998). The DVLO theory says that the stability of a colloidal system is determined by the sum of the electrical double layer repulsive and van der Waals attractive forces which the particles experience as they approach one another (Deryagin and Landau, 1941; Verwey and Overbeek, 1948). In practice, the repulsive forces can be greatly affected by changing the ionic strength of the dispersion medium. In this study at a calcium concentration of 136.3 mg/L there was dispersion instability, the ZP was -17.3 ± 0.20 mV at pH 4.0, -17.6 ± 0.21 mV at pH 7.0 and -17.9 ± 0.25 mV at pH 9.0, and, therefore, the colloids in the dispersion adhered together and formed visible aggregates. A conclusion, which can be drawn from these results, is that the HA's zeta potential increases with increasing calcium and magnesium concentrations.

2.3.3 Monitoring humic material in the presence of divalent cations

Total organic carbon (TOC) values obtained directly by sample combustion and infrared CO_2 detection and indirectly by UV spectroscopy at 254 nm (as described in materials and methods: total organic carbon and UV spectroscopy, respectively) were used as surrogates for HA quantifications. The effect of pH, concentration of divalent cations and sample filtration on TOC values, determined by both methods, is depicted in Figure 2.6 for calcium and in Figure 2.7 for magnesium.

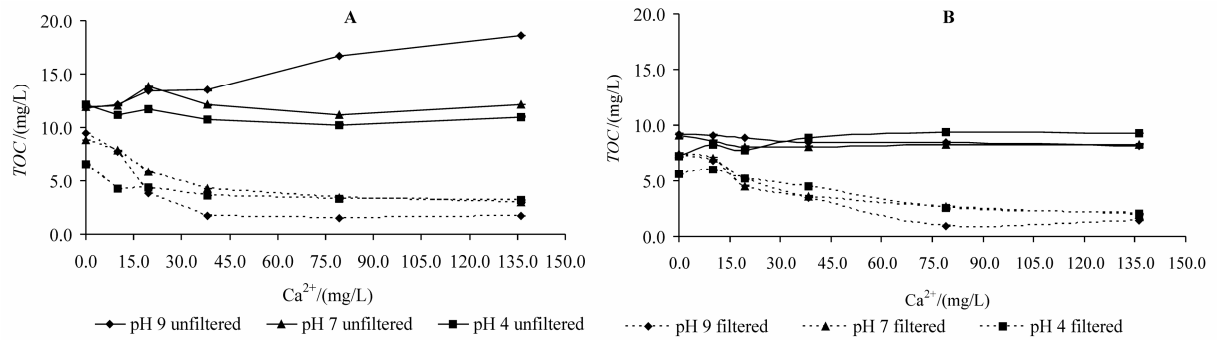

Figure 2.6 Effect of calcium on total organic carbon concentration of the HA suspension (10 mg/L of carbon) determined by: A) spectrophotometric method; B) combustion-infrared method.

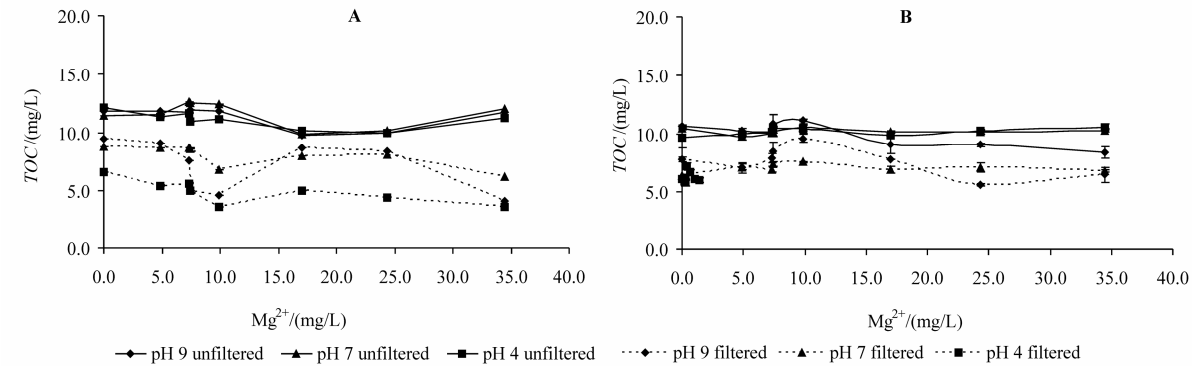

Figure 2.7 Effect of magnesium on total organic carbon concentration of the HA suspension (10 mg/L of carbon) determined by: A) spectrophotometric method; B) combustion-infrared method.

Control measurements carried out with pure solutions of calcium chloride and magnesium sulphate at the concentrations in question never yielded TOC values above 0.08 mg/L, so that direct influence of these substances in both methods was considered negligible. In most cases unfiltered samples presented considerably higher TOC concentrations than filtered ones independently of the method used, the concentration of divalent cations and the pH values. These results evidenced that a considerable portion of molecules were removed in the filtration process. Two hypotheses may explain the strong removal of organic carbon in the filtration process. The first hypothesis suggests that the presence of divalent cations enhances aggregation of HAs due to charge neutralization and functional group bridging; aggregates are more readily removed by filtration (Anđelković *et al.*, 2004). This hypothesis is corroborated by the observed increase of the zeta potential (became less negative) with increasing concentrations of calcium and magnesium (Fig. 2.4, 2.5) that indicated suspension instability. The second hypothesis is based on the adsorption of HA molecules onto the filtration membrane. As pointed out in several studies the adsorption of HAs increases with increasing concentrations of the metal ions, which shielded the electrostatic repulsion among HA molecules and thus facilitated their deposition on the membrane surface (Clark and Lucas, 1998; Jones and O'Melia, 2000; Wang *et al.*, 2001).

Differences in TOC results between spectrophotometric versus combustion-infrared methods were detected mainly for unfiltered samples, and specifically in the presence of calcium at both pH 7.0 and 9.0, which is in keeping with the different basic measurement principles. The principle of the spectrophotometer is based on Beer's law: the absorbance of a compound at a specific wavelength is proportional to the concentration of the compound (Skoog and Leary, 1988). This law is only valid

when all of the constituents in the sample have no chemical reaction between each other which was not the case in the present study. Calcium ions interacted with the HA and molecular aggregation was promoted with the consequent increase in turbidity, principally, at basic pH. The higher TOC results obtained by the spectrometric method might thus be explained by light scattering effects (Wang and Hsieg, 2001). The principle of the combustion-infrared method is the oxidation of organic carbon to CO_2 by combustion, therefore, the molecular structure as well as molecular interactions do not influence the TOC measurement.

It is important to stress that UV spectroscopy (254 nm) and combustion-infrared methods are based in different principles, while the first method measures the amount of aromatic structures (Kabsch-Korbutowicz, 2008), the second method quantifies carbon independently of the molecule's chemical structure. The use of UV spectroscopy to monitor natural organic matter in surface water is a fast and relatively inexpensive method that gives information about the aromatic content as well as the total organic content (when associated with a standard calibration curve). However, the results should be interpreted with care since the presence of calcium and magnesium ions and pH interfere with the analysis' results.

To compare both analytical methods, the errors associated with the determination of TOC of an unfiltered aqueous suspension of HA with a carbon concentration of 10 mg/L at pH 7.0 were compared. The results were 10.3 ± 2.0 mg/L for the combustion-infrared method and 11.91 ± 0.14 mg/L by the UV spectroscopy method. The combustion-infrared method gave the most accurate value although the precision was lower than the one obtained in the UV spectroscopy method. Moreover, the combustion-infrared method provided the least changes for unfiltered and filtered samples in the presence of divalent cations, at different pH values.

Surface water may present a highly variable composition regarding calcium (1.6 - 413.5 mg/L) and magnesium (1.6 – 259.2 mg/L) concentrations and pH values (2.5 - 8.2) (Claret *et al.*, 2003; MacLeod and Whitfield, 1996; Markich and Brown, 1998; Olías *et al.*, 2004). Thus, the combustion-infrared method is recommended for HA quantification in surface water.

2.4 CONCLUSIONS

From this work's results can be concluded that humic acids monitoring with respect to its total organic carbon content (TOC) by different methods leads to different results. Unfiltered samples presented considerably higher total organic carbon (TOC) values than filtered ones, independently of the method used. The higher differences of TOC values between unfiltered and filtered samples were

detected in the presence of calcium at pH 9.0 using the spectrophotometric method. The UV spectroscopic method gives information about the aromatic content of the sample, unlike the combustion infrared method, but suffers from interferences resulting from molecular aggregation in the presence of divalent cations. The combustion-infrared method gave the most accurate values although the precision was lower than the one obtained in the UV spectrophotometric method. In this regard and due the fact that pH values and divalent cations concentration in surface water may vary considerably, the combustion-infrared method is recommended for HSs quantifications in surface water.

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3 | CHARACTERIZATION OF BIOFILM FORMATION ON A HUMIC MATERIAL

Biofilms are major sites of carbon cycling in streams and rivers. Therefore, it is crucial to improve the knowledge about the biofilms' structure and microbial composition to understand their contribution in the self-purification of surface water. The present work intends to study the biofilm formation in the presence of humic substances (HSs) as a carbon source. Two biofilm flowcells were operated in parallel; one with synthetic river water, displaying a background carbon concentration of 1.3 ± 0.8 mg/L, the other with added HSs and an overall carbon concentration of 9.7 ± 1.0 mg/L. From the biofilms' results of culturable and total countable cells can be concluded that the presence of HSs did not significantly enhance the biofilm cell density. However, the biofilm formed in the presence of HSs presented slightly higher values of volatile suspended solids (VSS) and protein. One possible explanation for this result is that HSs adsorbed to the polymeric matrix of the biofilm and were included in the quantification of VSS and protein. The microbial composition of the biofilm with addition of HSs was characterized by the presence of bacteria belonging to beta-Proteobacteria, *Cupriavidus metallidurans* and several species of the genus *Ralstonia* were identified, and gamma-Proteobacteria, represented by *Escherichia coli*. In the biofilm formed without HSs addition beta-Proteobacteria, represented by the species *Variovorax paradoxus*, and bacteria belonging to the group Bacteroidetes were detected. In conclusion, the presence of HSs did not significantly enhance biofilm cell density but influenced the bacterial diversity in the biofilm.

Rodrigues, A.L., Brito, A.G., Janknecht, P., Silva, J., Machado, A.V., Nogueira, R., 2008. J. Ind. Microbial Biotechnol. 35: 1269-1276.

3.1 INTRODUCTION

Most of the microbial activity in streams resides in biofilms. Biofilms are microbial communities which have attached to surfaces and play key roles in carbon and nutrients cycling in streams (Lock, 1993; Dahm, 1981). High levels of natural organic matter (NOM) in surface water constitute a problem to the water industry because they are associated with the formation of carcinogenic disinfection by-products (e.g. trihalomethanes) upon chlorination of drinking water. Therefore, understanding the contribution of biofilms to regulate organic matter in water is important for the sustainable management of surface water resources. Moreover, stream biofilms might be exploited to improve surface water quality to be used in drinking water plants.

Humic substances (HSs) and non-humic substances correspond to the categories of NOM. HSs result from microbial degradation of organic matter, possibly followed by reactions of polymerization, condensation and oxidation. These reactions produce highly complex organic molecules (Beckett, 1990). HSs are mixtures characterized by the presence of aromatic and aliphatic compounds with mainly carboxylic and phenolic functional groups (Thurman, 1985). HSs, particularly humic and fulvic acids, typically account for the majority (about 75 %) of the dissolved organic carbon (DOC) in river water (Barreto *et al.*, 2003). Concentrations from 1 mg/L to 60 mg/L DOC are found in surface waters with typical values ranging from 2 mg/L to 10 mg/L (Beckett, 1990). The main DOC transformations occur in the riverbed (Dahm, 1981). Here, a complex assemblage of cells, mostly bacteria and fungi, embedded in a gelatinous matrix of exopolimeric substances (EPS) forms biofilms (Lock, 1993). The EPS offer potential binding sites for a variety of inorganic and organic compounds (Flemming, 1995) which can be uptaken by microorganisms.

The traditional model of dissolved organic matter (DOM) degradation assumes that simple organic molecules of low molecular weight (LMW) decompose faster than high molecular weight (HMW) compounds (Saunders, 1976). LMW compounds are less complex, thus they are considered more labile (Saunders, 1976). The more recent studies of Amon and Benner (1996) suggest a new conceptual model whereby the bioreactivity of organic matter decreases along a continuum of both size (from large to small) and diagenetic state (from fresh to old). This size-reactivity continuum model suggests that the bulk of HMW DOM is more bioreactive and less diagenetically altered than the bulk of LMW DOM and that degradation of organic matter in aquatic environments leads to formation of refractory LMW compounds. Sun *et al.* (1997) suggested that aliphatic carbon is the principal form of carbon being utilized by bacteria and that their ability to utilize DOM decreases as the aliphatic carbon content of the DOM decreases. In contrast with the "size-reactivity continuum model", the availability of DOC for bacteria is assumed to be controlled by the presence, abundance,

and steric accessibility of specific chemical functional groups rather than by molecular size. Recently, the work of Fischer *et al.* (2002) and Axmanovà *et al.* (2006) also supported the hypothesis that certain fractions of DOC might be readily utilized for bacterial growth irrespective of molecular size depending on the presence and abundance of specific chemical groups.

Composition of bacterial communities growing on HSs is poorly documented in literature. In general, beta-Proteobacteria have been described as a dominant group in eutrophic rivers (Araya *et al.*, 2003; Manz *et al.*, 1999). Manz *et al.* (1999) studied the composition of bacterial communities in mature river biofilms using fluorescence in situ hybridization and reported that alpha-Proteobacteria and the Bacteroidetes were prevalent over beta-Proteobacteria and gamma-Proteobacteria. In this context, the knowledge about the structure and function of biofilms growing on complex organic matter needs more consistent studies. In the present work, a model biofilm flowcell was developed to evaluate the contribution of humic substances to biofilm and suspended growth under controlled hydrodynamic conditions.

3.2 MATERIALS AND METHODS

3.2.1 Experimental system

A biofilm flowcell system was designed to study biofilms growing under defined hydrodynamic conditions (Figure 3.1).

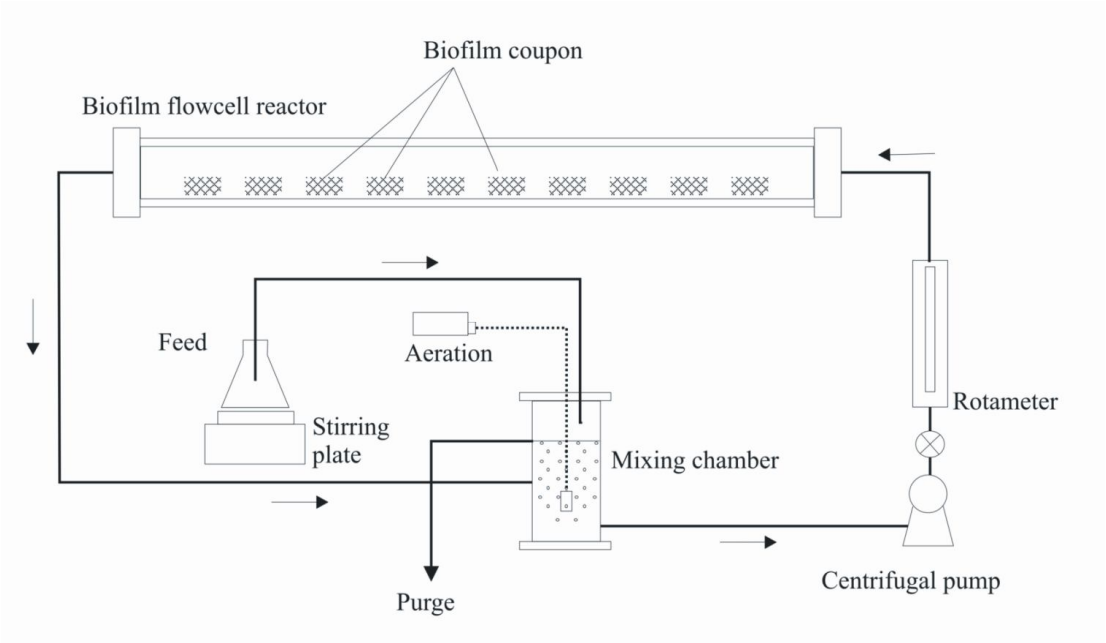

Figure 3.1 Schematic of experimental setup.

The media was recirculated from a mixing chamber to the flowcell and back. The cell was made of poliacrylic material with 0.042 m inner diameter and 1.04 m length, corresponding to a total submerged surface area of 0.14 m^2. The flow velocity was maintained at 0.04 m/s (laminar regime, Re = 1669). Ten independently removable thermopoly propylene coupons (Matala™) with 7.2 cm length, 2.5 cm width and 1 cm height in average were fitted in the cell, allowing for biofilm sampling. The coupons presented a highly porous tridimensional structure. The specific surface area of the material was 204 m^2/m^3, yielding an average surface of 2.74×10^{-4} m^2 per coupon.

3.2.2 Media

The synthetic river water was composed of 0.06 mg/L KH_2PO_4, 0.76 mg/L NH_4Cl, 0.67 mg/L KNO_3, 23.51 mg/L $MgSO_4.7H_2O$, 19.54 mg/L $CaCl_2.2H_2O$, 27.60 mg/L $MgCl_2.6H_2O$, 20.94 mg/L KCl, 1.88 mg/L $(NH_4)_2[Fe(SO_4)_2].6H_2O$, 3.87 μg/L $ZnSO_4.7H_2O$, 153.18 μg/L $MnCl_2.H_2O$, 3.18 μg/L $CuSO_4.5H_2O$, 0.14 ng/L $CoCl_2.6H_2O$, and 3.48 ng/L $NiCl_2.6H_2O$ (Markich and Brown, 1998). Humic substances (HSs), purchased from the International Humic Substances Society (IHSS), were extracted from Elliot Silt Loam Soil (BS102M) (Swift, 1996). A concentrated stock solution was prepared by dissolving 100 g of HSs in 1 L of NaOH solution, 4 g/L. This solution was mixed for 24 h and centrifuged at 5000 rpm for 30 min. The concentrated HSs stock solution (1 g/L of carbon) was stored in the dark at 4 °C (Ellis *et al.*, 2000). Distilled water was used in the preparation of all solutions.

3.2.3 Experimental approach

Two experiments were run in parallel under identical hydrodynamic conditions. The flowcell designated as test was fed with a humic material solution containing 9.7 ± 1.0 mg/L of carbon, while the control was fed with a synthetic river water mineral solution without humic material. Experiments were performed in duplicate. The synthetic river water presented a background carbon concentration of 1.3 ± 0.8 mg/L, originating from the distilled water used in the preparation. The feed to both flowcells was supplied at a rate of 0.25 mL/min. Temperature was maintained at 20 ºC by means of cold water circulation in the water jacket around the flowcell and the pH was adjusted, with 2 M NaCl and HCl solutions to 7.0 ± 0.2. The values of the experimental parameters flow velocity, carbon concentration, temperature, and pH were chosen as 0.04 m/s, 10 mg/L, 20 ºC, and 7.0, respectively, because they are representative of environmental conditions in several streams (Markich and Brown, 1998; Tank and Dodds, 2003). A microbial mixed culture from a sand filter treating surface river water (the water treatment plant from Braga, Portugal) was used as inoculum in both systems. Both flowcells were operated in the dark. During a period of 10 weeks, samples of biofilm and suspended biomass were collected weekly from both experiments and analyzed for volatile suspended solids (VSS), protein, culturable cells [namely, colony-forming unit (CFU)] and total countable cells (TC). Measurements were made in duplicate.

3.2.4 Sampling

The biofilm in the flowcells was sampled by removing a coupon and placing it aseptically into a falcon tube containing 40 mL of a sterile buffer solution (Ringer's solution). The tube was vigorously vortexed for 5 min, sonicated for 15 min in a sonication bath (Model SC-52) and vortexed again for 5 min. This procedure ensured maximum biofilm removal from the coupon. Subsequently the coupon was removed and the biofilm suspension was then homogenized for 20 min using a tissuemizer with SBS-dispensing tool (model AV 5). This procedure was indispensable for the subsequent cell enumeration after DAPI staining using an epifluorescence microscope. Suspension grab samples (40 mL) were taken aseptically in the recirculation tank and centrifuged at 4500 rpm for 10 min. The supernatant was rejected and the pellet was resuspended in 40 mL sterile buffer solution. The suspension was then subjected to a similar treatment as the biofilm suspension.

3.2.5 Analytical Methods

3.2.5.1 Total organic carbon (TOC)

Total organic carbon (TOC) was measured spectrophotometrically at 600 nm using the Method 10129 from Hach Lange GmbH. Organic carbon is oxidized with persulphate in the presence of acidic conditions and the carbon dioxide formed is captured by and indicator solution that changes color proportionally to the amount of organic carbon originally present in the sample. The results are expressed in mg/L of carbon.

3.2.5.2 Biomass quantification

Volatile suspended solids were measured according to the gravimetric method described in Standard Methods (APHA, 1998). Protein was measured according to the Lowry method (Lowry *et al.*, 1951), modified by Peterson (1977), using the Sigma protein assay kit (Sigma Diagnostics, St. Louis, Mo., USA). Culturable cell counts were determined by spread plating 100 µL of cell suspension on R2A agar medium and incubation at room temperature for 7 days. Samples for total cell counts, previously fixed with 40 g/L paraformaldehyde, were stained with 4,6-diamino-2-phenylindole (DAPI) (0.25 mg/L) for 15 min, filtered onto a black-stained polycarbonate filter (Nucleopore) with 2.27 cm^2 of filtration surface area and rinsed with 5 mL of sterilized distilled water. Cell enumeration was carried out in an epifluorescence microscope (Zeiss, filter set nº 01, excitation λ = 372 nm, emission λ = 456 nm) at 1000 fold magnification, a total of 20 fields was evaluated using a graduated grid divided into 346 squares (1×10^{-6} cm^2 each square). The total surface area examined per sample was 1.8×10^{-4} cm^2 (20 fields times 9 squares).

3.2.5.3 Biofilm community characterization

The composition of the bacterial community of 10-week-old biofilms was studied by 16S rRNA sequence analyses. Colonies from R2A plates presenting distinct morphologies and colors were selected and their purity was checked by successive plating in R2A plates. Subsequently the DNA was extracted and the 16S rRNA genes were amplified and sequenced. For DNA extraction a loop of fresh cells was placed into a tube containing 20 µL of alkaline lysis solution, composed of 0.25 % sodium dodecyl sulphate (SDS) and 0.05 M NaOH, and 180 µL distilled water. The tube was

vortexed during 1 min and then placed at 95 ºC during 5 min. After 5 min centrifugation at 14500 rpm the pellet was discarded and the supernatant stored at -20 °C until use. 1 µL of genomic DNA was used as template in a 25 µL reaction mixture consisting of 0.8 µM F8 (5´- AGA GTT TGA TCC TGG CTC AG -3´) and R537 (5´- TAT TAC CGC GGC TGC TGG CA -3´) primers (Fermentas), 200 µM of each deoxynucleoside triphosphate, polymerase chain reaction (PCR) buffer (2 mM $MgCl_2$), and 0.04 U of Taq polymerase (Fermentas). Thermal cycling was carried out with an initial denaturing step at 95 ºC for 5 min, followed by 40 cycles of denaturation at 95 ºC for 30 s, annealing at 55 ºC for 30 s, and elongation at 72 ºC for 45 s; cycling was completed by a final elongation step at 72 ºC for 10 min. A negative control (no DNA added) was included in all sets of amplification. The presence and size of the amplification product was determined by agarose gel (1.2 %) electrophoresis of the reaction product (5 µL). Ethidium bromide-stained bands were recorded with an Eagle Eye documentation system (Stratagene). Purification of the PCR product to remove excess primers and nucleotides was performed with shrimp alkaline phosphatase (2.0 U/µL) and exonuclease I (10.0 U/µL) (USB Corporation, Cleveland, Ohio). Briefly, 1 µL of each enzyme was added to 5 µL of PCR product, the enzymes were activated for 15 min at 37 °C, followed by inactivation at 80 ºC for 15 min. Cycle sequencing was performed using the Big Dye Terminator V3.1 Kit according to the manufacturer's protocol (Applied Biosystems). The labeled amplification product was analysed after removal of excess dyes on an ABI 310 Genetic analyzer (Applied Biosystems). The 16S rDNA sequences were assembled, edited, and compared to those in the GenBank. Sequences were aligned with MegAlign (DNASTAR Inc., Madison, WI, USA). Phylogenetic trees were computed with PAUP version 4.0b8 (Sinauer Associates Inc., Sunderland, MA, USA) using the neighbour-joining method and the Kimura two-parameter model for calculating distances. Nucleotide sequences were deposited in GenBank under the accession numbers: EU330532 through EU330542. Gaps were treated as missing data. Additional sequences were retrieved from GenBank.

3.2.5.4 Infrared spectroscopy

Fourier transform infrared (FTIR) spectra of HSs and 10-week-old biofilms formed with and without HSs were recorded with a ABB spectrometer (model FTLA 2000-104) using a spectral range varying from 4000 cm^{-1} to 500 cm^{-1} and a resolution of four; a minimum of five scans per spectrum was performed. Biofilms were removed from the coupons, as previously described. The biofilm suspension was centrifuged and the resulting pellet was dried at 60 ºC and finely ground to powder

(Quintelas *et al.*, 2008; Ramesh *et al.*, 2006). This procedure destroyed biofilms' structure. HSs were also dried at the same temperature. Twenty milligram of each biofilm powder and HSs were encapsulated in 200 mg of KBr in order to prepare translucent sample disks used for FTIR analysis. Background correction for atmospheric air was used for each spectrum.

3.2.6 Statistical analyses

A *t*-test was used, where specific means were being compared. Acceptance or rejection of the null hypothesis was based on a α-level of 0.05 in all cases.

3.3 RESULTS AND DISCUSSION

3.3.1 Dynamics of biofilm and suspended biomass development

The effect of HSs on biofilm growth was studied using the parameters VSS, protein, culturable and total countable cells. The results are depicted in Figure 3.2.

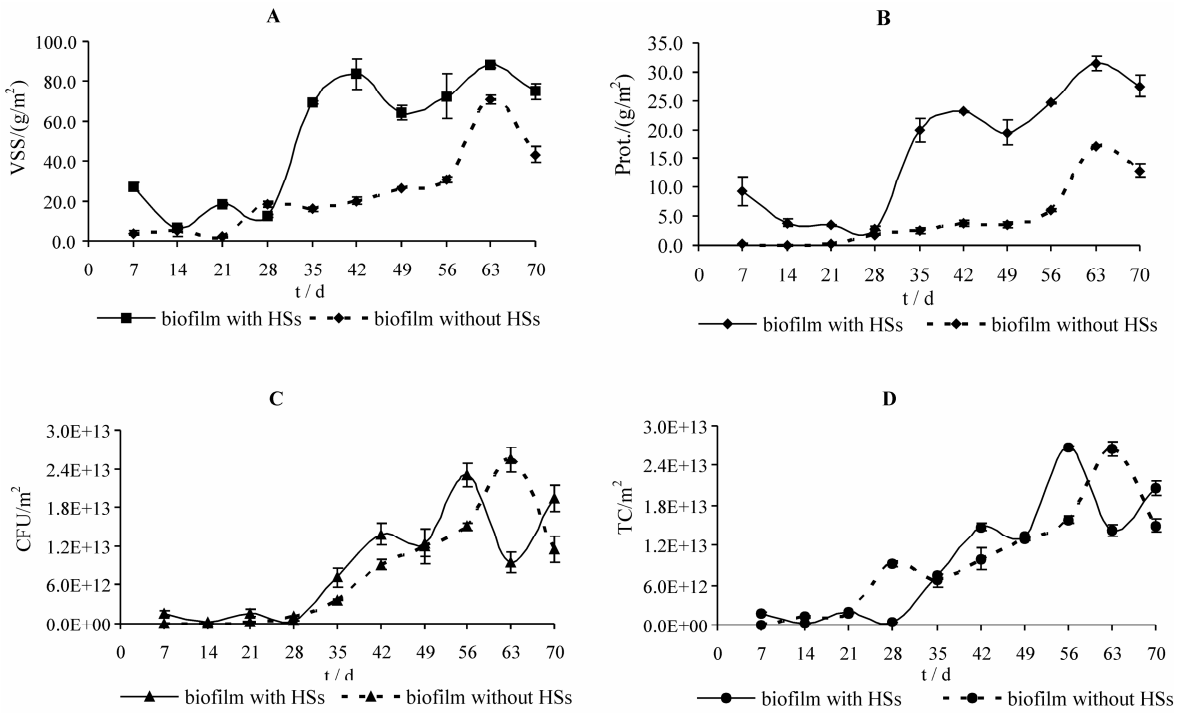

Figure 3.2 Biofilm growth over 10 weeks. The SSV, protein, culturable, and total countable cells per square meter are presented in panels *A*, *B*, *C* and *D*, respectively.

The values of VSS and protein (panels A and B, respectively) were significantly higher (*t*-test) in the biofilm formed in the presence of HSs than in their absence. However, the results of culturable and total countable cells (panels C and D, respectively) were not significantly different. In the presence of HSs (9.7 ± 1.0 mg/L carbon), the culturable and total countable cell values obtained at the end of the experiment were 1.93×10^{13} colony-forming unit CFU/m^2 and 2.06×10^{13} TC/m^2, respectively, while in the absence of HSs the values of the same parameters at the same time were of the same order of magnitude 1.15×10^{13} CFU/m^2 and 1.50×10^{13} TC/m^2. One possible explanation for this result is that HSs adsorbed to the polymeric matrix of the biofilm and were included in the quantification of VSS and protein. In fact, in the presence of HSs a gradual darkening of the biofilm was observed (from light yellow to brown) which might be indicative of HSs adsorption. This hypothesis is supported by Fein *et al.* (1999) who reported the adsorption of HSs to the bacterial

surface of a pure culture of *Bacillus subtilis* and by several other authors that described the adsorption of HSs to biofilms (Battin *et al.*, 1999).

According to the results and the above discussion, it seems that biofilm growth was not significantly affected by the presence of HSs. The background carbon concentration derived from the distilled water without HS addition contributed to the same amount of biofilm cell density as was observed in the presence of HSs. Few investigations have described the involvement of HSs in biofilm growth. Ellis *et al.* (2000) studied biofilm formation in three different carbon sources and reported that the substrate that provided the highest biofilm total cell density was amino acids, followed by carbohydrates, and humics.

Regarding the growth of suspended bacteria (Figure 3.3), no significant changes (*t*-test) were detected during the experimental lifetime. Thus the biofilm formation, relatively to suspended bacteria, was enhanced by biological, physicochemical and hydrodynamic experimental factors. This result is in agreement with those reported by Araya *et al.* (2003) and Crump *et al.* (1999) who found that respiring bacteria were higher in biofilms than in stream water samples.

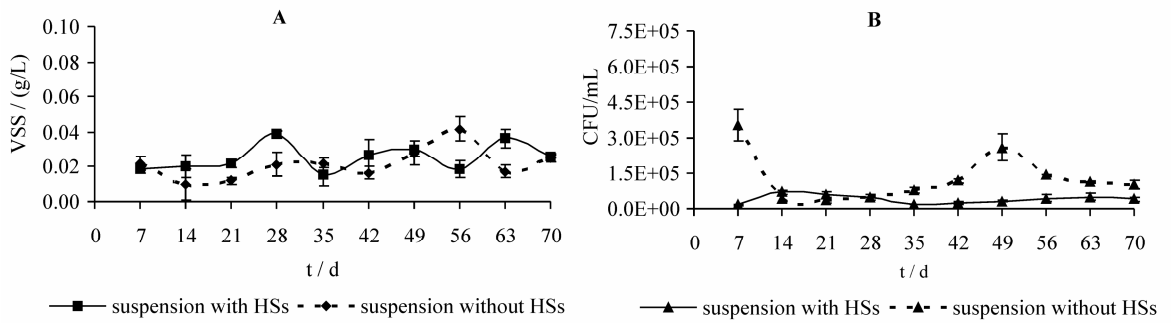

Figure 3.3 Suspended bacteria growth over 10 weeks. The suspended volatile solids and culturable cells are presented in panels *A* and *B*, respectively.

As pointed out in several studies culturable cells might be a small fraction of the total number of cells in a sample (Kisand *et al.*, 2002; Simu *et al.*, 2005). However, in the present work the numbers of TC were not significantly different from the culturable ones obtained either in the presence or absence of HSs. The culturability of bacteria in biofilms formed in the presence and absence of HSs was 94 and 77 %, respectively, obtained in the last week of the experiment. Similar high percentages of bacterial culturability are described in studies carried out with the R2A medium for mineral water [65 %, (Defives *et al.*, 1999) and sediment bacteria [82 %, (Foght *et al.*, 2004)]. This result might be explained by the fact that a nutrient poor media (R2A agar) was used for

bacteria cultivation in combination with a low incubation temperature (20 ºC) and an extended incubation time (7 days) (Reasoner, 2004).

3.3.2 Characterization of HS and biofilm composition by FTIR

To evaluate the presence of functional groups that might be responsible for HSs binding to biofilm, the FTIR spectra of HSs and biofilms formed both in the presence and absence of HSs were determined (Figure 3.4).

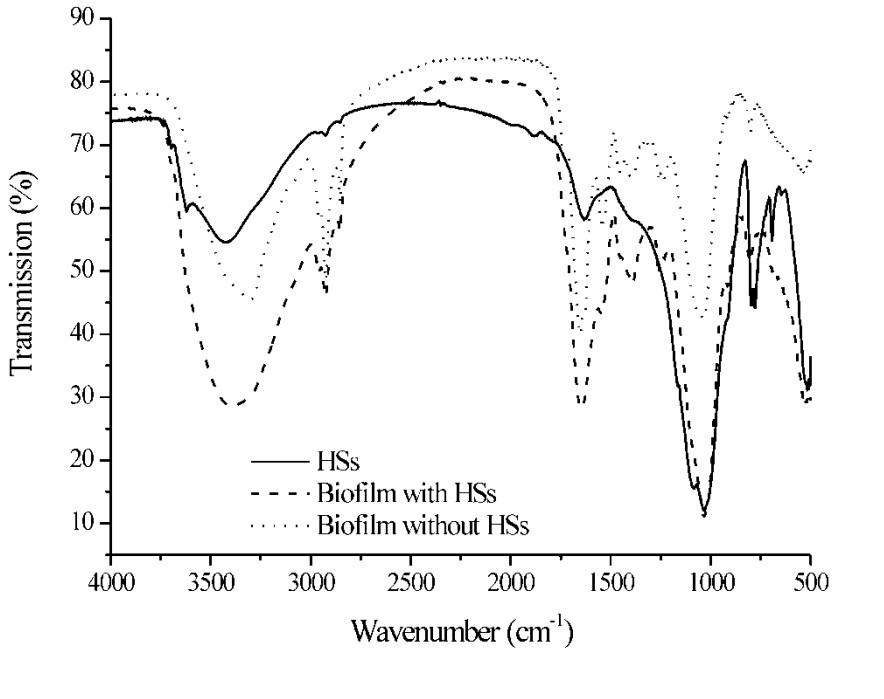

Figure 3.4 FTIR spectra of humic material and both biofilms formed on the presence and absence of humic material.

A high number of absorption peaks reveal the complex nature of both HSs and biofilms. The most important features of HSs spectrum are: (1) a broad band at 3600-3200 cm^{-1}, corresponding to H–bonded OH groups as well as H–bonded N–H groups, (2) a small peak at 2920 cm^{-1}, due to C–H stretching of alkyl structures, (3) a peak at 1628 cm^{-1}, corresponding to carbonyl groups C=O (e.g. amides), or stretching alkenes conjugated with or other double bonds C=C, and (4) a broad band at 1120-990 cm^{-1} with a sharp peak at 1031 cm^{-1}, associated to C–O stretching in polysaccharides and silicates. The presence of silicates is indicative of the soil nature of the HSs. The most important features of both biofilms' spectra are: (1) the large absorption band at 3500-3100 cm^{-1}, resulting

from –OH of hydroxyl groups (≈ 3400 cm^{-1}) and N–H stretching modes (amide A ≈ 3300 cm^{-1}), (2) the region between 3000 cm^{-1} and 2800 cm^{-1}, showing the C–H stretching vibrations of $–CH_3$, $–CH_2$ and –CH functional groups, which are characteristics of fatty acid chains of the various membrane amphiphiles (e.g. phospholipids) and of some amino acid side-chain groups, and (3) the region between 1800-1500 cm^{-1}, associated with amide bands, C=O, C–N and C=C stretching of the DNA or RNA heterocyclic base structures. A complex absorption can be observed between 1500 cm^{-1} and 1300 cm^{-1}, arising mainly from –CH2 and $–CH_3$ bending modes of lipids and proteins. The spectral region between 1200 cm^{-1} and 900 cm^{-1} is generally dominated by the symmetric stretching vibration of PO_2^- groups in nucleic acids and by a complex sequence of peaks associated with C–O–C and C–O–P stretching vibrations of various oligo and polysaccharides (Naumann, 2000). The spectrum of the biofilm formed with HSs shows differences in certain peaks relatively to the spectrum of the biofilm without HSs, namely in the region of 3500-3100 cm^{-1} (the signal at 3380 cm^{-1} became less pronounced), between 1800-1400 cm^{-1} (peaks at 1742 cm^{-1}, 1548 cm^{-1} and 1459 cm^{-1} almost disappear), and in the region of 1200-900 cm^{-1} (a new peak appears at 909 cm^{-1}). These differences observed between the two spectra, i.e. decrease of peaks' intensity and appearance of new peaks indicate changes in functional groups which might be related to the presence of HSs in the biofilm. Since FTIR analysis was performed in dehydrated and highly disrupted biofilms, differences between spectra cannot corroborate the hypothesis formulated previously concerning HSs adsorption to the biofilm.

3.3.3 Biofilm microbial composition

The biofilm community composition was based upon only those microorganisms that were culturable. The main reason to have chosen a cultivation-dependent method was related to the high percentage of bacterial culturability obtained in the present study. In the biofilm formed with HSs, evaluation of the microbial community composition by the 16S rRNA approach revealed the presence of bacteria belonging to the subclasses beta-Proteobacteria, *Cupriavidus metallidurans* and several species of the genus *Ralstonia* were identified, as well as gamma-Proteobacteria represented by *Escherichia coli*. A phylogenetic tree including all sequenced species is given in Figure 3.5. Beta-Proteobacteria are common in aquatic ecosystems (Araya *et al.*, 2003), drinking water biofilms (Schmeisser *et al.*, 2003), and soil (Chang *et al.*, 2007). The HSs used in the present study, extracted from Elliot Silt Loam Soil (IHSS), enhanced the growth of bacteria from the genus *Cupriavidus* and *Ralstonia* that are common in soil.

In the biofilm formed without HSs, the presence of beta subclass-Proteobacteria, represented by the species *Variovorax paradoxus*, and bacteria belonging to the group Bacteroidetes was detected. The genus *Variovorax* is common in soil and water environments and the group Bacteroidetes are well known to degrade complex macromolecules (Kojima *et al.*, 2006). Noble *et al.* (1990) reported that members of the group Bacteroidetes are adapted to low nutrient and substrate concentrations. Geller (1986) found that *Flavobacterium* isolated from lake water decompose refractory substrates more efficiently than other strains. Further research in biofilms formed on HSs should be carried out to establish a relationship between the structure and the function of the microbial communities (Romaní *et al.*, 2004). Culture-dependent and independent methods used to assess microbial diversity in the same sample have been shown to produce distinct results (Tamaki *et al.*, 2005). Thus the presence and abundance of the retrieved sequences within the cells present in the original environmental sample should be estimated by hybridization with nucleic acid probes.

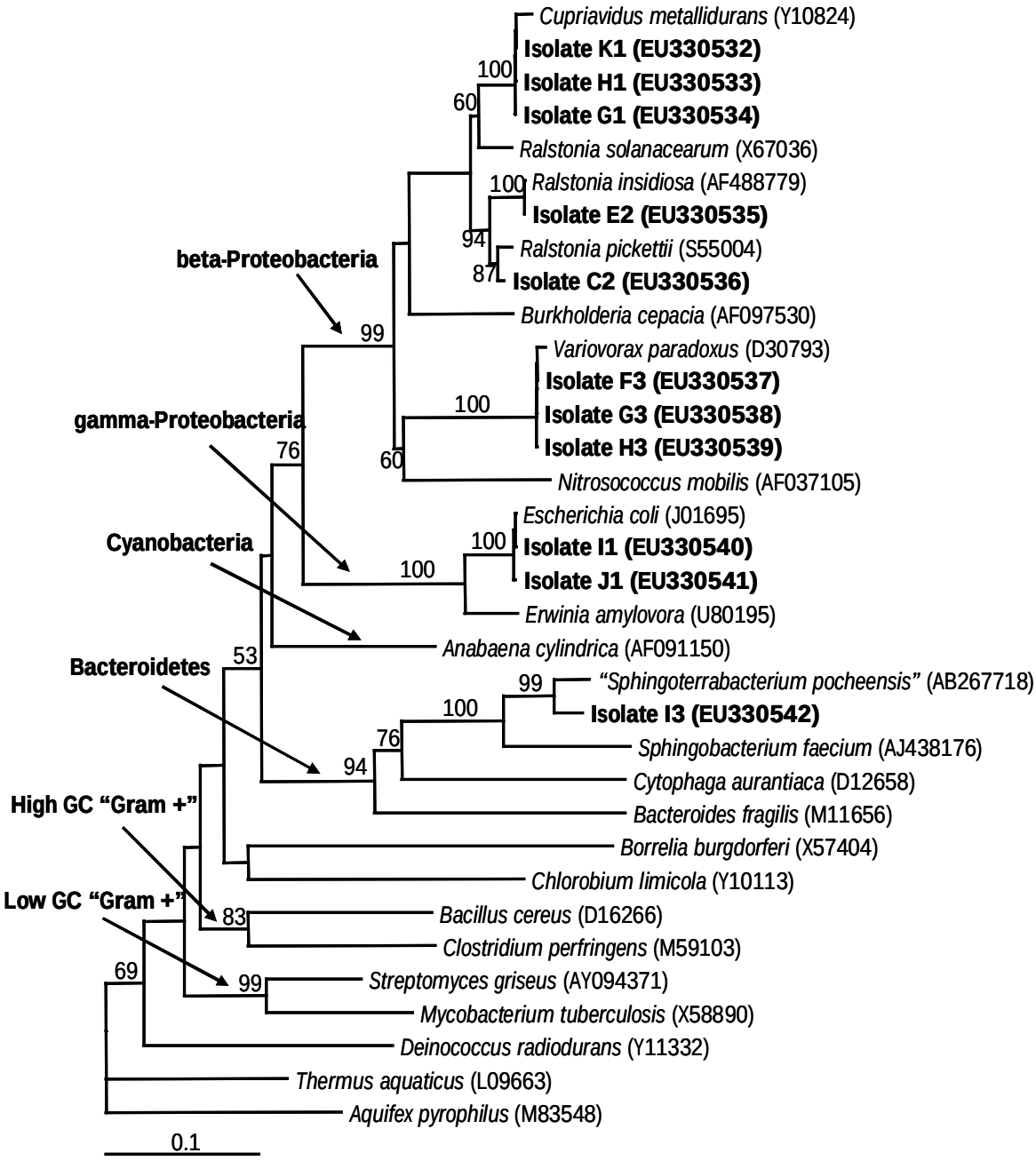

Figure 3.5 Phylogenetic tree of representative isolates and of selected species of bacteria obtained by neighbor-joining analysis of 16S rRNA gene, partial sequence, using PAUP 4.0b8. The numbers given on the branches are the frequencies (>50 %) with which a given branch appeared in 1000 bootstrap replications. *Aquifex pyrophilus* was used as outgroup. Sequences determined by the authors of the present study are typed in *boldface*. Additional sequences were retrieved from GenBank (species names followed by the corresponding accession number between parentheses).

3.4 CONCLUSIONS

The results of the flowcell operated with HSs and without HSs (9.7 ± 1.0 mg/L and 1.3 ± 0.8 mg/L of carbon concentration, respectively), at flow velocity of 0.04 m/s and 20 °C, do not indicate positive effects of the presence of humic material on biofilm cell density. Investigations will be carried out to establish the nature and magnitude of biofilm response to specific controlled changes in stream conditions specifically fow velocity, DOC, and oxygen.

The presence of HSs, however, did influence biofilm microbial composition: biofilm formed in the presence of HSs was characterized by the occurrence of beta-Proteobacteria belonging to the genus *Cupriavidus* and *Ralstonia*, while the presence of beta-Proteobacteria belonging to the genus *Variovorax paradoxus* and Bacteriodetes were found in the biofilm formed in the absence of HSs.

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4 BIOFILMS FORMED ON HUMIC SUBSTANCES: RESPONSE TO FLOW CONDITIONS AND CARBON CONCENTRATIONS

Microbial activity in streams and rivers occurs preferentially in biofilms that are exposed to dynamic conditions of flow velocity and organic carbon availability. Thus, the aim of this study was to investigate the response of biofilms formed with and without humic substances (HSs) to an increase in flow velocity (0.04 to 0.10 m/s) and HSs concentration (9.7 ± 1.0 to 19.8 ± 0.4 mg/L of C). Three biofilm flow cells were operated with and without HSs (1.3 ± 0.8 mg/L background C). The highest amount of biofilm, measured as volatile suspended solids and total countable cells, was observed at 0.10 m/s without HSs. HSs concentration had no significant impact on biofilm cell density under different flow velocities. The bacterial community composition of the biofilm with HSs was characterized by sequences with high similarities (≥ 97 %) to the genus *Dokdonella* (gamma-Proteobacteria) and to the genera *Comamonas*, *Cupriavidus* and, *Ralstonia* (beta-Proteobacteria). Sequences retrieved from the biofilm without HSs presented high similarities (≥ 97 %) to the genus *Sphingomonas* (alpha-Proteobacteria) and the genus *Nitrosospira* (beta-Proteobacteria). Experimental results suggested that the presence of HSs under different concentrations and flow velocities did not significantly enhance the cell density of biofilms but influenced its microbial composition.

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4.1 INTRODUCTION

Most bacteria in streams and rivers live in biofilms attached to surfaces which contribute to carbon and nutrients cycling (Lawrence and Neu, 2004; Fukuda *et al.*, 2006). The excess of natural organic matter (NOM) in water is associated with the formation of carcinogenic disinfection by-products (e.g. trihalomethanes) upon the chlorination step in drinking water plants. Therefore, scientific studies are required to understand the role of biofilms on organic matter regulation and self-purification of stream water. Besides, stream biofilms could be applied to improve surface water quality to be used in drinking water plants.

NOM is mainly formed by humic substances (HSs). They are complex organic molecules containing various functional groups (e.g., carboxylic and phenolic) and, randomly condensed aromatic rings (Stevenson, 1994; Lin *et al.*, 2001). HSs, particularly humic and fulvic acids, make up about 75 % of the dissolved organic matter (DOC) in stream waters (Wetzel, 2001; Hertkorn *et al.*, 2002). The question as to whether or not HSs are taken up by organisms has been argued intensely in the literature. Although they have commonly been considered recalcitrant (e.g. Moran and Hodson 1990; Leff and Meyer 1991), Frazier *et al.* (2005) and Kim *et al.* (2006) recently demonstrated that humic substances substantially contribute to stream biodegradable DOC. The main DOC transformations take place in the streambed (Lawrence and Neu, 2004).

Biofilm formation is controlled by an array of coupled physical, chemical, and biological processes, including: (1) cell transport to the substratum, (2) cell adhesion and attachment, (3) biofilm formation by cellular growth and extracellular polymer production, (4) biofilm detachment and (5) substrate and product flux to and from the biofilm (Applegate and Bryers, 1990; Bryers and Characklis, 1982). Literature studies reported that hydrodynamic conditions and biodegradable organic material concentration are two of the most important factors that affect biofilm growth and detachment in natural systems (Battin *et al.*, 2003; Lawrence *et al.*, 2004; Romaní *et al.*, 2006; Besemer *et al.*, 2007). Phylogenetic affiliation of bacteria in biofilms has been investigated by molecular 16S rRNA gene-targeting techniques. Microbial community fingerprinting by denaturing gradient gel electrophoresis (DGGE), cloning and, sequencing analysis was shown to be a suitable cultivation-independent techniques for analysis of complex microbial communities (Amann *et al.*, 1995). Typical groups of bacteria that are present in freshwater biofilms are affiliated to alpha-Proteobacteria, beta-Proteobacteria and gamma-Proteobacteria. Generally, alpha-Proteobacteria dominate natural freshwater systems that are oligo- or mesotrophic (Pinhassi and Berman, 2003;

Olapade and Leff, 2005). Beta-Proteobacteria have been reported to be the most abundant bacterial group in freshwater ecosystems and are also considered a diverse and opportunistic group present in abundance in a variety of conditions, namely in polluted rivers (Brümmer *et al.*, 2003; Manz *et al.*, 1999). The majority of freshwater ammonia-oxidizing bacteria are also beta-Proteobacteria (Purkhold *et al.*, 2003). Gamma-Proteobacteria may be an opportunistic group under high-nutrient conditions, presenting a lower activity in oligotrophic systems (Pinhassi and Berman, 2003). They also contain some of the common bacteria which are known as pollutant degraders (Uta *et al.*, 2005) and some of the main aquatic denitrifiers (Gómez-Villalba *et al.*, 2006). Several studies reported that the type and quantity of organic matter as well as hydrodynamic conditions may influence the abundance and composition of bacteria in stream ecosystems (Leff and Meyer, 1991; Koetsier *et al.*, 1997; Leff, 2000; Battin *et al.*, 2003).

Stream biofilms are exposed to a wide range of flow velocities and carbon concentrations. The range of flow velocities described in literature varies between 0.019 and 0.29 m/s (Koserski, 2003; Tank and Dodds, 2003). In oligotrophic surface waters, DOC concentrations reported are between 1 to 60 mg/L (Beckett, 1990). So far, little is known about the effect of dynamic changes of hydrodynamics and carbon availability on the cell density and bacterial community composition of biofilms formed on HSs. Therefore, to investigate the effects of flow velocity and carbon concentration of HSs on biofilm structure and microbial composition a model biofilm flowcell was used (Rodrigues *et al.*, 2008).

4.2 MATERIALS AND METHODS

4.2.1 Experimental system

A biofilm flowcell system was designed to study biofilm formation with and without humic substances (HSs) under defined hydrodynamic conditions (Rodrigues *et al.*, 2008). Briefly, the biofilm flowcell system contained a mixing chamber connected to a flowcell and the liquid phase was recirculated from the mixing chamber to the flowcell and back using a centrifugal pump. The recirculation flow provided by the centrifugal pump was maintained constant by means of a rotameter. Synthetic river water supplemented with HSs was continuously added to the mixing chamber at a constant flow rate of 0.25 mL/min, using a peristaltic pump, and the mixing chamber was continuously aerated to provide aerobic conditions in the system. The dissolved oxygen in the system was around 7 mg/L. The biofilm flowcell system was operated in the dark and the

temperature was maintained at 20±1 °C by means of cold water circulation in the water jacket around the flowcell. The flowcell was made of polyacrylic material with 0.042 m inner diameter and 1.04 m length, corresponding to a total submerged area of 0.14 m^2. Ten independently removable thermopoly propylene coupons (Matala™) with 0.072 m length, 0.025 m width and 0.010 m height in average were fitted in the cell, allowing for biofilm sampling. The specific surface area of the material was 204 m^2/m^3, yielding an average surface of 2.74×10^{-4} m^2 per coupon.

4.2.2 Experimental approach

Synthetic river water was prepared as previously described (Rodrigues *et al.*, 2008) and used in the experiments. The background carbon concentration of the synthetic river water was 1.3 ± 0.8 mg/L, resulting from the distilled water used in the preparation of all solutions. The synthetic river water was supplemented with a concentrated HSs solution and the final pH adjusted to 7.0 ± 0.2 with either 2 M NaCl or HCl solutions. HSs extracted from Elliot Silt Loam Soil (BS102M) were purchased from the International Humic Substances Society (IHSS). A concentrated HSs stock solution (1 g/L carbon) was prepared and stored in the dark at 4 °C.

Biofilms were previously formed during 10 weeks with (9.7 ± 1.0 mg/L of carbon) and without (1.3 ± 0.8 mg/L of carbon) HSs, at a flow velocity of 0.04 m/s (Rodrigues *et al.*, 2008). Three biofilm flowcell systems were run in parallel, two with HSs and one without. To study the biofilm's response to variations in flow velocity and HSs concentration, four experimental assays were carried out, as described in Table 4.1. All experiments were performed over a four weeks period. The biofilm´s response to an increase in HSs concentration from 9.7 ± 1.0 mg/L to 19.8 ± 0.4 mg/L of carbon and flow velocity from 0.04 m/s to 0.10 m/s was evaluated. The velocity was calculated as the ratio between the flow rate and the cross sectional area of the empty flowcell. An increase in flow velocity was achieved by manipulating the recirculation flow rate in the flowcell.

Table 4.1 Experimental conditions used in the flowcells to study the biofilm's response to changes in humic substances (HSs) concentration and flow velocity.

Biofilm	Mode of operation		Flowcell
	Carbon concentration (mg/L)	Flow velocity (m/s)	
Without HSs	1.3 ± 0.8	0.04 → 0.10	A
With HSs	9.7 ± 1.0 → 19.8 ± 0.4	0.04	B
	9.7 ± 1.0	0.04 → 0.10	Ca
	9.7 ± 1.0 → 19.8 ± 0.4	0.10	Cb

4.2.2.1 Biofilm's response to an increase in flow velocity

The experiment was conducted in two subsequent steps. During the first one, which is not represented here, flowcells **A** and **Ca** were operated at a flow velocity of 0.04 m/s (200 L/h) during 10 weeks without (1.3 ± 0.8 mg/L of carbon) and with (9.7 ± 1.0 mg/L of carbon) HSs, respectively (Rodrigues *et al.*, 2008). Subsequently, the flow velocity in both biofilm flowcells was increased from 0.04 m/s to 0.10 m/s (499 L/h), and maintained at this last value during 4 weeks.

4.2.2.2 Biofilm's response to an increase in HSs concentration under low and high flow velocities

During the first step of the experiment, which is not represented here, the flowcell **B** was operated at a flow velocity of 0.04 m/s during 10 weeks with HSs (9.7 ± 1.0 mg/L of carbon). Subsequently, the HSs concentration was increased from 9.7 ± 1.0 mg/L to 19.8 ± 0.4 mg/L, and maintained at this last value during 4 weeks.

To evaluate the biofilm's response to an increase in HSs concentration at a flow velocity of 0.1 m/s, a third experimental step was carried out using flowcell Ca, whose experimental conditions are indicated in Table 4.1 under the denomination of flowcell **Cb.** The HSs concentration was increased from 9.7 ± 1.0 mg/L to 19.8 ± 0.4 mg/L, and maintained at this last value during 4 more weeks.

4.2.3 Sampling

Biofilm samples were collected weekly for volatile suspended solids (VSS) and total countable cells (TC). Measurements were made in duplicate. For DNA extraction samples were collected before and after each change in HSs concentration and flow velocity.

The biofilm in the flowcells was sampled by removing a coupon and placing it aseptically into a falcon tube containing 40 mL of a sterile buffer solution (Ringer's solution). The tube was vigorously vortexed for 5 min, sonicated for 15 min in a sonication bath (Model SC-52) and vortexed again for 5 min. Subsequently, the clean coupon was removed and the biofilm suspension was then homogenized for 20 min using a tissuemizer with SBS-dispensing tool (model AV 5).

4.2.4 Analytical methods

Total organic carbon was determined by sample combustion and infrared carbon dioxide detection (5310 B) according to Standard Methods (APHA *et al.*, 1998) using a Shimadzu TOC-5000A analyzer. Biomass was quantified both as volatile suspended solids (VSS) and total countable cells (TC). VSS were measured according to the gravimetric method described in Standard Methods (APHA *et al.*, 1998). Samples for TC, previously fixed with 40 g/L paraformaldehyde, were stained with 4,6-diamino-2-phenylindole (DAPI) (0.25 mg/L) for 15 min, filtered onto a black-stained polycarbonate filter (Nucleopore) with 2.27 cm^2 of filtration surface area and, rinsed with 5 mL of sterilized distilled water. Cell enumeration was carried out in an epifluorescence microscope (Zeiss, filter set nº 01, excitation λ = 372 nm, emission λ = 456 nm) at 1000 fold magnification, a total of 20 fields was evaluated using a graduated grid divided into 346 squares (1×10^{-6} cm^2 each square). The total surface area examined per sample was 1.8×10^{-4} cm^2 (20 fields times 9 squares).

4.2.5 Molecular characterization of biofilm´s bacterial community

16S rRNA gene-targeting techniques, such as denaturing gradient gel electrophoresis (DGGE), cloning and, sequencing analysis were used as cultivation-independent tools for analysis of bacterial diversity in the biofilms.

4.2.5.1 DNA extraction and amplification

Approximately 2 mL of a well homogenized biofilm sample was frozen at the time of sampling and stored at -20 ºC. Total genomic DNA was extracted using a UltraClean™ Soil DNA Isolation Kit (MO BIO, Carlsbad, CA, USA) according to the protocol described by the manufacturer.

The 16S rRNA genes were amplified by polymerase chain reaction (PCR) using a *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA) with primers targeting conserved domains. PCR amplification was performed in a 50 mL reaction mixture containing 5 mL of 10X PCR buffer (20 mM Tris-HCl (pH 8.4), 500 mM KCl), 3 mM $MgCl_2$, 200 mM of each of the four deoxynucleoside triphosphates (dNTP), 1.25 U of *Taq* polymerase, 200 nM of each primer and 1 mL of appropriately diluted template DNA. All primers used were synthesized by STAB Vida (Oeiras, Portugal). For DGGE analysis the 16S rRNA genes were amplified using suitable bacterial primers U968-GC-f (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAACG CGAAGAACCTTAC-3') and L 1401-r (5'-CGGTGTGTACAAGACCC-3'), targeting the V6 to V8 region (Muyzer *et al.*, 1993; Nübel *et al.*, 1996). The termocycling program used for amplification was: an initial denaturation step of 5 min at 95 ºC; 35 cycles of denaturation at 95 ºC for 30 s, annealing at 56 ºC for 40 s and, elongation at 72 ºC for 90 s; and post-elongation at 72 ºC for 5 min. The reactions were subsequently cooled to 4 ºC. The 16S rRNA genes were also amplified for cloning using the forward primer Bact27-f (5'–AGAGTTTGATCMTGGCTCAG–3') and the universal primer Uni1492-r (5'–ACGCCTACCTTGTTACGACTT-3') (Heuer *et al.*, 1997). The program of amplification was similar to the one described above but with 25 cycles and an annealing temperature of 52 ºC. A negative control (no DNA added) was included in all sets of amplification. The size of the PCR products was estimated using a 100 bp DNA ladder (Frilabo, Porto, Portugal) by electrophoresis in a 1 % (wt/vol) agarose gel stained with ethidium bromide.

4.2.5.2 DGGE analysis

Denaturing gradient gel electrophoresis analysis of the amplicons was carried out using the Dcode system (Bio-Rad, Hercules, CA, USA) in gels containing 8 % (w/vol) polyacrylamide (37.5:1 acrylamide/bis-acrylamide). Denaturating gels were generated using a gradient mixer and a peristaltic pump by standard procedures. The gradient was made at an approximate rate of 4 mL/min. A linear denaturant gradient of 30 to 60 % was used for all analyses, where a denaturing

strength solution of 100 % was defined as 7 M urea (Sigma-Aldrich, St. Louis, MO, USA) and 40 % formamide (Fluka Chemie, Buchs, Switzerland) (McCaig et al, 2001). Gels were run for 16 h at 85 V in a 0.5X TAE buffer (50X Tris acetate: 242 g Tris, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA (pH 8.0) per liter) at 60 ºC. Subsequently, DGGE gels were stained with silver as previously described by Sanguinetty *et al.* (1994).

4.2.5.3 Cloning and sequencing of PCR amplified products

PCR amplicons were purified with the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and cloned into *E. coli* JM 109 (Invitrogen, Carlsbad, CA, USA) using the Promega pGEM-T Easy vector system I (Promega, Madison, WI, USA), according to the manufacturer's instructions. The transformed cells were plated onto LB (Luria-Bertani) medium (10 g of Bacto-Triptone, 5 g of Bacto-yeast extract, 5 g of NaCl, 15 g Bacto agar, pH 7.0) containing ampicillin (100 mg/mL), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside: 80 mg/mL) and, IPTG (Isopropyl β-D-1-thiogalactopyranoside: 0.5 mM) to identify white-colored recombinant colonies. 50 white-coloured clones were collected and screened by DGGE. PCR was carried out on the cell lysates using the primer pair above described for DGGE analysis. The DGGE mobility of amplicons was compared to the band-pattern of the biofilm. The clones whose amplicon's corresponded to bands in the biofilm community profile were selected for sequencing. Clones with sequences showing identical DGGE mobility were also selected for replicate sequencing. Plasmids of selected clones were purified using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and subjected to DNA sequence analysis. Sequencing reactions were performed at STAB Vida using SP6 (5' – ATT TAG GTG ACA CTA TAG – 3') and T7 (5'- TAA TAC GAC TCA CTA TAG G – 3') sequencing primers. The sequence information was imported into the BioEdit v7.0.9 software package (Hall, 1999) for assembly. Consensus sequences obtained were manually checked and corrected when necessary. They were also checked for potential chimera artefacts by the CHECK_CHIMERA program of the Ribosomal Database Project II (http://35.8.164.52/cgis/chimera.cgi?su= SSU). Similarity searches for the 16S rRNA gene sequences derived from the biofilm clones were performed using the NCBI Blast search program within the GenBank database (http://www.ncbi.nlm.nih.gov/blast/).

Sequence data of the 16S rRNA genes have been deposited in the GenBank database under accession numbers GU169052 to GU169067.

4.2.6 Statistical analyses

A *t*-test was used where specific means were compared. Acceptance or rejection of the null hypothesis was based on a α-level of 0.05 in all cases.

4.3 RESULTS AND DISCUSSION

4.3.1 Effects of flow velocity and HSs concentration on biofilms

This study was designed to investigate the response of biofilms to an increase in HSs concentration and flow velocity. Biofilms were previously formed during 10 weeks with (9.7 ± 1.0 mg/L of carbon) and without (1.3 ± 0.8 mg/L of carbon) HSs, at a flow velocity of 0.04 m/s (Rodrigues *et al.*, 2008). The composition of biofilms formed under these conditions was characterized by a VSS content of 36.1 ± 1.7 g/m^2 with HSs (flowcell Ca) and 34.3 ± 1.7 g/m^2 without HSs (flowcell A). The TC content of both biofilms was 1.2 × 10^{12} ± 1.0 × 10^{11} cell/m^2 with HSs and 1.4 × 10^{12} ± 0.2 × 10^{11} cell/m^2 without HSs. The VSS and TC content of both biofilms were very similar in spite of the different concentrations of soluble organic carbon in solution. Therefore, soluble organic carbon from HSs and distilled water might have not been equally bioavailable.

Figure 4.1, panels I and II, shows the VSS and TC per square meter in biofilms without HSs (flowcell A) and with (flowcell Ca) HSs.

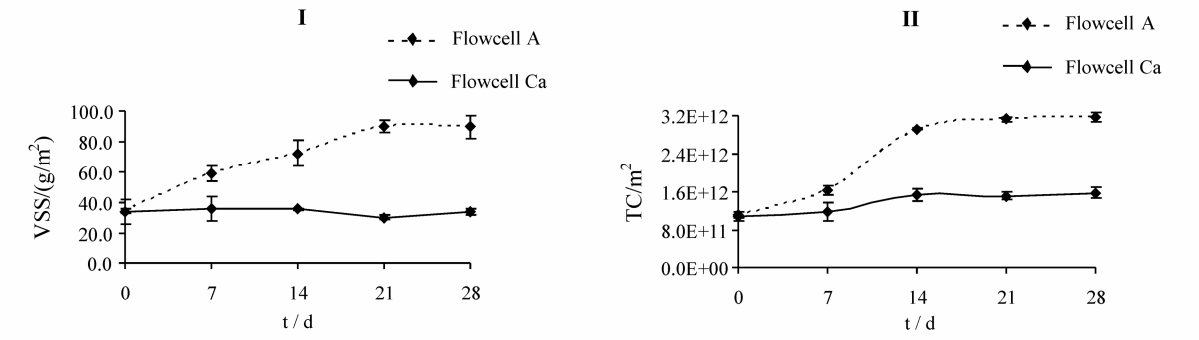

Figure 4.1 Biofilm response to an increase in flow velocity from 0.04 m/s, at time zero, to 0.10 m/s, after 4 weeks, without (flowcell A - 1.3 ± 0.8 mg/L of C) and with (flowcell Ca - 9.7 ± 1.0 mg/L of C) HSs. Volatile suspended solids (VSS) and total countable cells (TC) per square meter are presented in panels I and II, respectively.

The flow velocity in both biofilm flowcells was increased from 0.04 m/s to 0.10 m/s, at time zero, and maintained at this last value during 4 weeks. These results revealed that flow velocity had no significant effect on the VSS content in the biofilm with HSs (flowcell Ca); VSS varied from 36.1 ± 1.7 g/m^2 to 33.9 ± 1.7 g/m^2 after 4 weeks. Conversely, the VSS content in the biofilm without HSs (flowcell A) increased significantly from 34.3 ± 1.7 g/m^2 to 89.7 ± 5.1 g/m^2 (circa 62 %). Regarding the TC content, both biofilms were significantly impacted by velocity but the effect was considerably higher in the biofilm without HSs. The TC content of the biofilm without HSs increased circa 55 % in 4 weeks (from 1.4 × 10^{12} ± 0.2 × 10^{11} cell/m^2 to 3.2 × 10^{12} ± 0.9 × 10^{11} cell/m^2), whereas in the biofilm with HSs a smaller increase was observed, circa 25 % (from 1.2 × 10^{12} ± 1.0 × 10^{11} cell/m^2 to 1.6 × 10^{12} ± 1.2 × 10^{11} cell/m^2). These results suggested that the impact of velocity in the composition of biofilms is positive and higher in the biofilm without HSs. Several studies reported that biofilms formed at higher velocities (turbulent conditions) presented higher bacterial numbers than those formed at lower velocities (laminar conditions) using a low concentration of biodegradable organic carbon, around 0.5 mg/L (Eriksson, 2001; Percival *et al.*, 1999; Ollos *et al.*, 2003; Lehtola, 2006; Simões *et al.*, 2006; Paris *et al.*, 2007). The explanation presented for these results is that the availability of organic carbon (growth limiting substrate) in the biofilm increased due to a lower mass transfer resistance in the liquid film boundary layer on top of the biofilm occurring at higher flow velocities.

Figure 4.2, panels I and II, shows the response of biofilms under low (0.04 m/s, flowcell B) and high (0.10 m/s, flowcell Cb) flow velocities to an increase in HSs concentration from 9.7 ± 1.0 mg/L to 19. 3 ± 0.4 mg/L of carbon, at time zero, and maintained at this last value during 4 weeks. Although VSS content of both biofilms might have not reached steady-state after 4 weeks, these results suggested that the VSS content of the biofilm at 0.04 m/s slightly increased, based on initial and final values (from 26.4 ± 2.9 mg/L to 31.3 ± 1.0 mg/L), whereas an opposite trend was observed in the case of the biofilm at 0.10 m/s (from 33.9 ± 1.7 mg/L to 21. 4 ± 0.3 mg/L). The TC content of the biofilm presented a similar trend. The combined effect of HSs concentration and flow rate, expressed as a mass flow rate, on biofilm's SSV and TC is depicted in Figure 4.3. The results suggested that the biofilm VSS and TC contents increased with an increase in the HSs mass flow from 2 g/h to 5 g/h. The highest amount of biofilm was observed at a flow velocity of 0.10 m/s and a HSs concentration of 9.7 mg/L. Doubling the mass flow rate from 5 g/h to 10 g/h produced a decrease of both VSS and TC in the biofilm. This result might be associated to the increase in the HSs concentration from 9.7 mg/L to 19.8 mg/L because flow velocity was maintained at 0.1 m/s.

As pointed out in several studies, HSs might form complexes with enzymes present in the biofilm matrix reducing its hydrolytic activity (Boavida and Wetzel, 2002; McNamara and Leff, 2004).

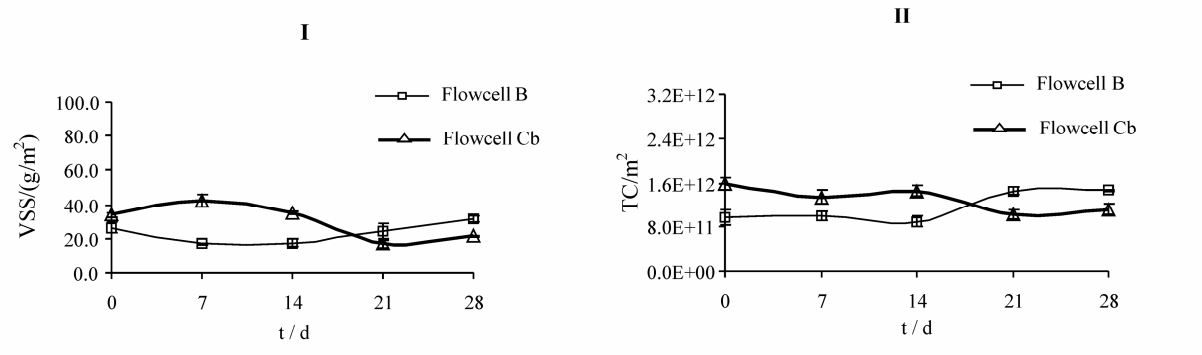

Figure 4.2 Biofilm response to an increase in HSs carbon concentration from 9.7 ± 1.0 mg/L to 19.8 ± 0.4 mg/L with flow velocities of 0.04 m/s (flowcell B) and 0.10 m/s (flowcell Cb). Volatile suspended solids (VSS) and total countable cells (TC) per square meter are presented in panels I and II, respectively.

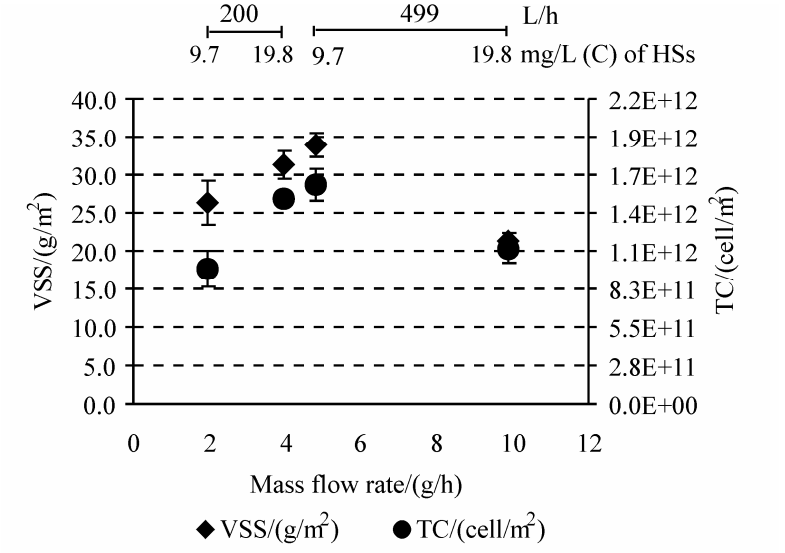

Figure 4.3 Biofilm response to an increase in HSs mass flow rate.

4.3.2 Effects of flow velocity and HSs concentration on biofilm´s bacterial community structure

The study of diversity and dynamics of bacteria in biofilms was based on DGGE patterns of partial 16S rRNA gene amplicons. DNA extracted from biofilms collected from each flowcell (A, B, Ca and Cb), at the beginning and end of each operation period, was used as template for amplification of the V6-V8 bacterial region. These amplicons were separated by DGGE and the obtained band patterns are depicted in Figure 4.4. Apart from changes in the relative intensity of a few bands, DGGE band-patterns of biofilms exposed to different flow velocities and carbon concentrations did not differ significantly from the initial ones (time 0). In the biofilm without HSs, a significant increased in the relative intensity of ribotypes represented by bands Ab1 and Ab2 was observed when the flow velocity was changed from 0.04 m/s (A_0) to 0.10 m/s (A_4), suggesting a positive effect of flow velocity in the predominance of these ribotypes. Regarding the biofilm with humic substances, besides an apparent decrease of the relative predominance of the ribotype represented by band P4 in the DGGE profile B_4 (19.8 ± 0.4 mg/L of C) when comparing to B_0 (9.7 ± 1.0 mg/L of C), no major shifts were detected suggesting that increasing the concentration of HSs did not influence remarkably the microbial community diversity (measured as number of bands).

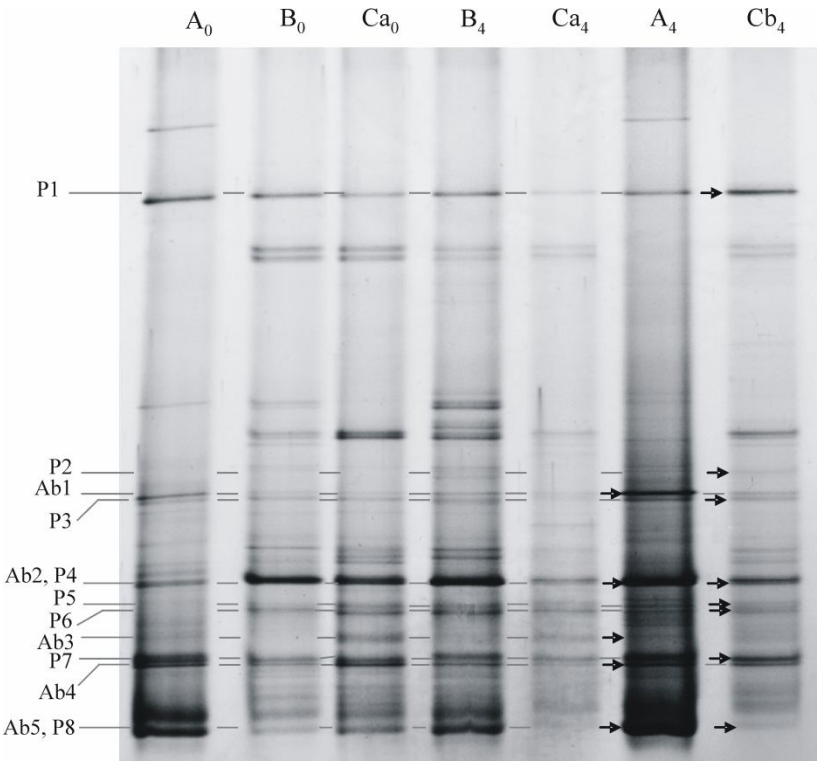

Figure 4.4 DGGE patterns of bacterial communities in biofilms without (A_0, A_4) and with (B_0, Ca_0, B_4, Ca_4, Cb_4) HSs under different hydrodynamic conditions and carbon concentrations: A_4 – after flow velocity increase of A_0, B_4 – after carbon concentration increase of B_0, Ca_4 – after flow velocity increase of Ca_0, Cb_4 – after flow velocity and carbon concentration increase of Ca_0. Annotated DGGE bands were further identified by cloning and sequencing.

To get an insight into the identity of the bacterial community represented in the DGGE patterns, 16S rRNA-genes of two representative biofilm samples without (A_4) and with (Cb_4) HSs were amplified, cloned and sequenced. The DGGE mobility of amplicons obtained from a total of 100 clones was compared to DGGE profiles of both biofilms to determine the fragments to which they match. A total of 16 clones were selected from both biofilms without (Ab1 to Ab7) and with (P1 to P9) HSs. Clones Ab1, Ab2, Ab4 and Ab5 presented bands that corresponded to the prominent ones (i.e., higher signal intensity) in the DGGE profile of the biofilm without HSs (A_4) and clones P1, P4 and P7 corresponded to the prominent bands in the one from the biofilm with HSs (Cb_4) (Fig. 4.4). Clones Ab6, Ab7 and P9 did not match any visible bands. Sequencing and blast searching of the selected bacterial clones resulted mainly in matches with unknown and uncultured microorganisms assigned to the Proteobacteria (clones Ab1, Ab2, Ab3, Ab4, Ab7, P2, P4, P7, P5, P6), Deinococcus-Thermus group (clones Ab5, P8) and, Acidobacteria (P1). In addition, close relatives to *Sphingomonas sp.*

(clone Ab6) belonging to the alpha subclass of Proteobacteria and close relatives to *Comamonas sp.* (clone P3) and *Cupriavidus pauculus* (clone P9) belonging to the beta subclass of the same group, were also found. The sequencing results are summarised in Table 4.2. Clones P4 and Ab2 presented similar band positions. Their sequences were most similar to those of yet uncultured microorganisms assigned to the gamma subclass of Proteobacteria. This subclass represents a collection of microorganisms that are diverse in their habitats, lifestyles and metabolic preferences (Mrázek *et al.*, 2006). Members of the gamma-Proteobacteria did not constitute a dominant phylogenetic group in stream biofilms or stream water, although their abundance in biofilms was about twice that in stream water (Araya *et al.*, 2003). Clones P7 was related to *Dokdonella sp.* and clone Ab4 to *Nitrosospira sp.*, which belong to different subclasses of Proteobacteria, namely gamma-Proteobacteria and beta-Proteobacteria, respectively. *Dokdonella*-like microorganisms are usually found in soils and have been associated to organic pollutants removal in wastewater treatment (Yoon *et al.*, 2006; Lin *et al.*, 2008), while *Nitrosospira*-like are ammonia-oxidizing bacteria (AOB) which have been detected in a variety of soil, marine, estuarine and freshwater systems and are crucial for the removal of nitrogen compounds in waste-water treatment plants (Painter, 1986, Avrahami *et al.*, 2007). Clones P8 and Ab5 presented similar band positions and show very low similarity to sequences deposited in the GenBank database. Clone Ab1 was closely related to subclass gamma-Proteobacteria (bacterium Ellin339). The bacterium Ellin339 was also detected in soils (Sait *et al.*, 2002).

Table 4.2 Sequencing results of the clones of biofilms formed without and with humic substances (HSs).

Clone	GenBankAccession number	Sequence length (bp)	Closest relatives (% sequence similarity)
Ab1	GU169052	1462	Bacterium Ellin339 (99%)
Ab2	GU169053	1470	Uncultured bacterium clone FAC50 (98%), Gamma proteobacterium KIS3-4 (98%)
Ab3	GU169054	1464	Uncultured ammonia-oxidizing bacterium (98%), *Nitrosospira sp.* Nsp65 (98%)
Ab4	GU169055	1460	Uncultured ammonia-oxidizing bacterium (99%), *Nitrosospira sp.* Nsp65 (99%)
Ab5	GU169056	1415	Uncultured Thermaceae bacterium clone HAVOmat118 (93%), *Meiothermus cerbereus* GY-5 (91%)
Ab6	GU169057	1411	S*phingomonas sp.* MG49 (99%)
Ab7	GU169058	1405	Uncultured bacterium clone 3BH-11EE (97%), *Caulobacter sp.* (95%)
P1	GU169059	1466	Uncultured bacterium clone 3BH-6HH (94%), *Geothrix fermentans* (94%)
P2	GU169060	1462	Bacterium Ellin307 (98%)
P3	GU169061	1446	*Comamonas sp.* PND-3 (97%)
P4	GU169062	1469	Uncultured bacterium clone FAC50 (99%), Gamma proteobacterium KIS3-4 (99%)
P5	GU169063	1463	Uncultured *Ralstonia sp* . clone GI6-1-F10 (99%), *Ralstonia sp* . EF1 (99%)
P6	GU169064	1461	Uncultured bacterium clone Pia-s-4 (97%), Denitrifying bacterium W99 (97%)
P7	GU169065	1473	Uncultured bacterium clone KD5-12 (97%), *Dokdonella sp.* KIS28-6 (97%)
P8	GU169066	1416	Uncultured Thermaceae bacterium clone HAVOmat118 (93%), *Meiothermus cerbereus* (90%)
P9	GU169067	1457	*Cupriavidus pauculus* (98%)

Comparative analysis of the sequences corresponding to prominent ribotypes in the DGGE profiles suggested that the prevalent groups of bacteria present in both biofilms formed with and without HSs are related to bacteria known to survive under low-nutrient conditions and/or in the presence of complex organic matter. Additional sequences retrieved only from biofilms formed without HSs were related to subclasses alpha-Proteobacteria (*Sphingomonas* and Caulobacteraceae family) and beta-Proteobacteria (*Nitrosospira* genus). These genera-like microorganisms have the capacity to survive in oligothrophic habitats (Gomila *et al.*, 2005; Srinivasan *et al.*, 2008) and to degrade a large variety of organic substrates (Poindexter, 2006; Pang and Liu, 2006). Other sequences retrieved only from biofilms with HSs were related to subclasses beta-Proteobacteria (*Comamonas*, *Cupriavidus* and *Ralstonia* genera) and gamma-Proteobacteria (bacterium Ellin337 and *Dokdonella* genus). The different sequences retrieved from both biofilm samples can not assure different bacteria diversity, however the DGGE profiles of both samples indicate different bacterial population's dominance in biofilms formed in the presence and absence of HSs.

The results obtained in this work showed a higher microbial diversity than the observed in the previous work (Rodrigues *et al.*, 2008), in which the carbon concentration and flow velocity corresponded to the initial ones of flowcell A and flowcell B. The differences observed might be due to three main reasons. Firstly, different methods (culture-dependent and culture-independent) were used to assess bacterial phylogenetic diversity and from literature it is known that culture-independent methods revealed greater microbial diversity than culture-dependent ones (Ward *et al.*,

1990). Secondly, flow velocity, source and concentration of NOM may influence biofilm composition (Koetsier *et al.*, 1997; Leff, 2000; Besemer *et al.*, 2007). Thirtly, biofilms had different ages.

4.4 CONCLUSIONS

From this study it can be suggested that HSs concentration had no significant impact on biofilm cell density under different flow velocities but HSs presence influenced biofilm microbial composition. The bacterial community composition of the biofilm with HSs was characterized by sequences with high similarities (≥ 97 %) to the genus *Dokdonella* (gamma-Proteobacteria) and to the genera *Comamonas*, *Cupriavidus* and, *Ralstonia* (beta-Proteobacteria) while sequences retrieved from the biofilm without HSs presented high similarities (≥ 97 %) to the genus *Sphingomonas* (alpha-Proteobacteria) and the genus *Nitrosospira* (beta-Proteobacteria).

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5 | CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

5.1 CONCLUSIONS

The research presented in this dissertation aimed to elucidate the influence of humic substances on biofilm formation.

In order to quantify HSs in surface waters and its involvement on biofilm growth, it was essential to investigate the molecular scale characteristics of HSs. Therefore, this research began (Chapter 2) by characterizing and quantifying humic acids (HAs) which were used to simulate HSs in surface waters. The HAs analysis indicated soil origin, high molar ratio of proton on saturated carbon atoms, as well as the presence of amines, amides, alcohols, phenols and carboxylic acids. The stability of HAs colloidal system was influenced by the presence of divalent cations, it decreased with increasing the concentration of divalent cations. This study also showed that HAs quantification, for filtered and unfiltered samples in the presence of divalent cations at different pH values, by UV spectrophotometric method and combustion infrared method yielded different results. The combustion infrared method gave the most accurate values and provided the least changes between filtered and unfiltered samples in the presence of divalent cations, at different pH values being, therefore, more suitable for HSs quantifications in surface water.

In the second study (Chapter 3) was investigated the biofilm formation on a humic material under a low flow velocity (0.04 m/s). The results indicated that biofilm growth was not significantly affected by the presence of HSs at 20 ºC. In fact, the background carbon concentration present in the distilled water without HSs addition contributed to the same amount of biofilm cell density as was detected in the presence of HSs (1.3 ± 0.8 mg/L and 9.7 ± 1.0 mg/L of carbon concentration, respectively). However, the presence of HSs affected the biofilm microbial composition (which identification was based on culture-dependent method). Biofilm bacteria formed in the presence of HSs was characterized by the occurrence of beta-Proteobacteria, *Cupriavidus metallidurans* and several species of the genus *Ralstonia* were identified, and gamma-Proteobacteria, represented by *Escherichia coli*. On the other hand, in the biofilm formed without HSs bacteria belonging to beta-Proteobacteria were detected, represented by the species *Variovorax paradoxus*, and bacteria belonging to the group Bacteroidetes.

In the final study of this dissertation (Chapter 4), which start with the final conditions of the previous work, it was evaluated the response of biofilms to an increase in HSs concentration and the flow velocity. The results indicated that the impact of velocity in the growth of biofilms was positive and higher in the biofilm formed in the absence of HSs than in its presence. These results also suggested that organic carbon derived from distilled water and HSs were not equally bioavailable. The better response of biofilm growth, under low (0.04 m/s) and high (0.10 m/s) flow velocities to

an increase in HSs from 9.7 ± 1.0 mg/L to 19.3 ± 0.4 mg/L of carbon was achieved, at a flow velocity of 0.10 m/s and a HSs concentration of 9.7 mg/L. Probably, this can be due to the fact that HSs form complexes with enzymes present in the biofilm matrix reducing its hydrolytic activity. Regarding the study of diversity and dynamics of bacteria using DGGE technique, in the biofilm without HSs was observed a significant increased in the relative intensity of two ribotypes (bands Ab1 and Ab2) when the flow velocity was changed from 0.04 m/s to 0.10 m/s. This examination suggested a positive effect of flow velocity in the predominance of these ribotypes. On the other hand, in the biofilm with HSs no major shifts were detected suggesting that increasing the concentration of HSs did not influence considerably the microbial community diversity.

The prevalent groups of bacteria (identified using culture-independent method) present in both biofilms formed with and without HSs are related to bacteria usually found in environments with low nutrient conditions and/or with the presence of complex organic matter. In fact, HSs concentration had no considerable impact on biofilm cell density under different flow velocities but influenced biofilm microbial composition. The biofilm with HSs was characterized by sequences with high similarities (≥ 97 %) to the genus *Dokdonella* (gamma-Proteobacteria) and to the genera *Comamonas*, *Cupriavidus* and, *Ralstonia* (beta-Proteobacteria). Although, sequences retrieved from the biofilm without HSs presented high similarities (≥ 97 %) to the genus *Sphingomonas* (alpha-Proteobacteria) and the genus *Nitrosospira* (beta-Proteobacteria). The microbial biofilm diversity determined using culture-independent method (Chapter 4) was higher than using culture-dependent method (Chapter 3). Three main reasons might explain the differences: the use of different methods of bacterial phylogenetic assessment; the influence of flow velocity and concentration of organic matter; and finally the different ages of biofilms.

5.2 SUGGESTIONS FOR FUTURE WORK

The research presented in this dissertation did not indicate a positive influence of HSs on biofilm growth which means that, in the studied conditions, biofilm application do not represent yet a biological solution for HSs removal from rivers. In fact, this research indicated some of the complexities of the relationship between HSs and bacteria, however the reason why biofilm growth was not enhanced in the presence of HSs remains unknown. Thus, additional research into biofilm metabolic responses to the presence of humic substances is needed (e.g., evaluation of enzymatic activity and quantification/identification of extracellular polysaccharides). It is also recommended to

carry out investigations to establish the magnitude of biofilm response in the presence of HSs to changes in stream conditions regarding the oxygen concentration that may vary between 0.0 mg/L to 18.0 mg/L. The identification of microbial populations represents an important tool to evaluate biofilm development. However, culture-dependent or culture-independent techniques for bacteria identification have limitations. It would be interesting to compare in the same study both methods in order to allow a more appropriate assessment of bacterial diversity.