Integrated Master in Bioengineering

Microalgal biofilm formation and nutrient removal

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“Give a man a fish and you feed him for a day. Teach a man to fish and you feed him for a lifetime”

(Chinese proverb)

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Abstract

In the last decades there has been an increase of nutrients input in water courses, due to industrialization, urbanization, agriculture and human practices, causing eutrophication and putting in risk the availability and quality of water resources, human health and the ecosystem's integrity. For many years, microalgae have been used to treat wastewater, but the high harvesting costs, associated with their microscopic dimensions, make their use not feasible. However, in the past few years, microalgal biofilms have emerged as a possible alternative, as they attach to a surface, making their recovery easier and more cost-effective than suspension-based systems.

The aims of this study were: (i) to understand the effect of surface physicochemical properties of selected microorganisms (*Chlorella vulgaris*, *Pseudokirchneriella subcapitata*, *Synechocystis salina* and *Microcystis aeruginosa*) and surfaces (polystyrene, PS; stainless steel 316, SS 316; poly(methyl metacrylate), PMMA; glass, G; polyvinyl chloride, PVC; and copper, Cu) on microbial adhesion; (ii) to study the effect of different surfaces and media composition (OECD test medium and synthetic domestic effluent) on biofilm formation by microalgae and cyanobacteria; and (iii) to optimize nutrients removal from culture medium using different biofilm reactors (a stirred tank reactor and a rotating disk reactor).

The free energy of hydrophobic interaction showed that both microalgae and cyanobacteria had a hydrophilic character. Conversely, SS 316 and PMMA are hydrophilic, whereas PS, PVC, G and Cu are hydrophobic surfaces. Also, a good correlation between free energy of hydrophobic interaction and free energy of adhesion ($R^2 \geq 0.986$) was obtained. The biofilm formation occurred during the first 24 h and followed the order PVC > SS 316 > PS > PMMA > G > Cu. Moreover, adhesion was higher when Organization for Economic Co-operation and Development (OECD) test medium was used as culture medium. Comparison between the thermodynamic approach and the laboratory assays showed that prediction of microbial adhesion depends on many factors not included on this approach. Regarding the reactor systems used for nutrients removal, the rotating disk reactor demonstrated to have a better performance than the stirred tank reactor. Also, higher stirring speeds allowed higher nitrogen and phosphorus removal efficiencies (81 and 97%, respectively), whereby European Union legislation limits for discharged effluents were overcome.

Key-words: Adhesion, Cyanobacteria; Microalgae; Microalgal biofilms; Nitrogen and phosphorus removal; Wastewater treatment.

Resumo

Nas últimas décadas tem havido um enriquecimento em nutrientes nos cursos de água, devido à industrialização, urbanização, agricultura e práticas humanas, causando eutrofização e colocando em risco a disponibilidade e qualidade dos recursos hídricos, saúde humana e integridade do ecossistema. Há já vários anos que as microalgas têm sido utilizadas no tratamento de águas residuais, mas os elevados custos associados à sua recolha, que estão também relacionados com as suas dimensões microscópicas, fazem com que as microalgas não sejam uma alternativa viável para este fim. Contudo, nos últimos anos, os biofilmes de microalgas têm surgido como uma possível alternativa, uma vez que as microalgas aderem a uma dada superfície, tornando a sua recolha do meio mais fácil e mais rentável, comparativamente a sistemas que têm por base microalgas em suspensão.

Os objetivos deste estudo foram: (i) perceber o efeito das propriedades físico-químicas de microrganismos selecionados (*Chlorella vulgaris*, *Pseudokirchneriella subcapitata*, *Synechocystis salina* and *Microcystis aeruginosa*) e de superfícies (poliestireno, PS; aço inoxidável 316, SS 316; polimetilmetacrilato, PMMA; vidro, G; cloreto de polivinil, PVC; e cobre, Cu) na adesão microbiana; (ii) estudar o efeito das superfícies e da composição do meio de crescimento (meio OECD e efluente doméstico sintético) na formação de biofilme pelas microalgas e cianobactérias; e (iii) otimizar a remoção de nutrientes do meio de cultura usando diferentes reatores de biofilme (um reator perfeitamente agitado e um reator de biodiscos).

A energia livre de interação hidrofóbica demonstrou que, tanto as microalgas como as cianobactérias, apresentaram um carácter hidrofílico. Por outro lado, o SS 316 e o PMMA são hidrofílicos, enquanto que o PS, PVC, G e o Cu são superfícies hidrofóbicas. Além disso, obteve-se uma boa relação ($R^2 \geq 0.986$) entre a energia livre de adesão e a energia livre de interação hidrofóbica. A formação de biofilme ocorreu nas primeiras 24 h e seguiu a ordem PVC > SS 316 > PS > PMMA > G > Cu. Aliás, a adesão foi maior quando se utilizou como meio de cultura o meio OECD. A comparação entre o modelo termodinâmico e os ensaios laboratoriais mostrou que para prever a adesão microbiana é necessário ter em consideração muitos outros fatores que não foram incluídos neste modelo. Em relação aos sistemas de reatores utilizados para a remoção de nutrientes, o reator de biodiscos demonstrou ter um desempenho melhor do que o reator perfeitamente agitado. Além disso, velocidades de agitação maiores permitiram uma maior eficiência de remoção de azoto e fósforo (81 e 97%, respetivamente), pelo que os limites da legislação da União Europeia para os efluentes que são descarregados foram ultrapassados.

Palavras-chave: Adesão, Cianobactérias; Microalgas; Biofilmes de microalgas; Remoção de azoto e fósforo; Tratamento de águas residuais.

Table of Contents

List of Figures

List of Tables

Nomenclature

ATS	Algal turf scrubber
CFU	Colony forming unit
CO_2	Carbon dioxide
Cu	Copper
EDS	Energy dispersive spectrometry
EPS	Extracellular polymeric substances
FDA	Fluorescein DiAcetate
G	Glass
N	Nitrogen
OECD	Organization for Economic Co-operation and Development
OD	Optical density
P	Phosphorus
PBR	Photobioreactor
PS	Polystyrene
PMMA	Poly(methyl methacrylate)
PVC	Polyvinyl chloride
RABR	Rotating algal biofilm reactor
RDR	Rotating disk reactor
SE	Synthetic effluent
SEM	Scanning electron microscopy
SS 316	Stainless steel 316
STR	Stirred tank reactor

Measure units

k	Nutrients uptake rate (mg L^{-1} d^{-1})
$C(t)$	Time-course evolution of nutrients concentration
$C(t_0)$	Nutrients concentration in the beginning of the experiment (mg L^{-1})
$C(t_f)$	Nutrients concentration at the end of the experiment (mg L^{-1})
γ_s^{LW}	Lifshitz van der Waals component of the surface tension (mJ m^{-2})

γ_s^{AB}	Acid-base component of the surface tension (mJ m^{-2})
γ_s^-	Electron donor component of the surface tension (mJ m^{-2})
γ_s^+	Electron acceptor component of the surface tension (mJ m^{-2})
ΔG_{sws}^{TOT}	Free energy of hydrophobic interaction or hydrophobicity (mJ m^{-2})
$\Delta G_{adhesion}$	Free energy of adhesion (mJ m^{-2})
θ_B	Contact angle using α-bromonaphthalene (º)
θ_F	Contact angle using formamide (º)
θ_W	Contact angle using water (º)
λ	Lag time (d)
μ	Specific growth rate (d^{-1})

CHAPTER 1

1. Work outline

1.1. Background and project presentation

Nowadays, pollution associated problems are starting to be a major societal concern [1]. The industrialization, rapid economic development, agricultural practices, expansion of water networks and humans daily life routines have led to an increased input of nutrients and consequent eutrophication of water bodies, putting in risk the quality of water resources and the ecosystem's integrity [1-3]. Additionally, there are still many cases where the wastewater is released directly, without any treatment, into water systems. Therefore, there is a need for new alternatives for the treatment and safe discharge of wastewaters into water bodies [1].

In this sense, microalgae have gained attention as they are an economically viable and environmentally friendly solution. Using microalgae for wastewater treatment has many advantages, since they can grow under very hard conditions and, by using carbon dioxide (CO_2) and light as carbon and energy sources, they can effectively remove nutrients, especially nitrogen (N) and phosphorus (P), the main chemical elements associated to the eutrophication of water bodies [4, 5].

However, microalgal harvesting is still a challenge. The microalgal small size and low density make their recovery from the effluent a limiting step [6]. Since algal biomass removal is essential for water discharge, new alternatives have been developed. Accordingly, microalgal biofilms emerged as a viable and eco-friendly solution, since, when in biofilm, microalgal cells are attached to a surface, making easier their removal from the treated effluent [7]. Furthermore, in the past few years, great nutrients removal efficiencies have been reported for microalgal biofilm-based systems, showing that they constitute an important alternative for wastewater treatment.

1.2. Main objectives

One of the most important objectives of this work was to evaluate the biofilm formation ability of the microalgae *Chlorella vulgaris* and *Pseudokirchneriella subcapitata* and the cyanobacteria *Synechocystis salina* and *Microcystis aeruginosa.*

To evaluate the ability of these microorganisms to form biofilm, an initial screening was performed to find the most appropriate materials for the selected microorganisms. For this, the characterization of surface physicochemical properties of the referred microorganisms and of different materials (polystyrene, PS; stainless steel 316, SS 316; poly(methyl methacrylate), PMMA; glass, G; polyvinyl chloride, PVC; and copper, Cu) was assessed through the measurement of contact angles. The free energy of adhesion based on a thermodynamic approach was then determined as a prediction of the interaction between the selected microorganisms and materials. Furthermore, *in vitro* assays were performed with the previously analysed microorganisms and materials, in order to quantify the microbial ability to form biofilm on the different surfaces. The effect of media composition on biofilm development was also assessed. Then, the obtained results were compared to *in vitro* adhesion experiments and the relationship between surface physicochemical properties and microalgal/cyanobacterial adhesion was evaluated.

Another aim of this study was the optimization of nutrients removal, in particular, nitrogen and phosphorus, from the culture media by using microalgal biofilms. Towards nutrients removal, two different biofilm-based systems were used: (i) a stirred tank reactor (STR); and (ii) a rotating disk reactor (RDR), operating in batch mode. In these experiments, both biofilm formation and nutrients removal was evaluated along the cultivation time. Additionally, light intensity, temperature, pH and dissolved oxygen concentration were daily monitored to observe their influence on nutrients removal efficiency.

1.3. Thesis organization

This work is divided in six chapters. Chapter 1 presents the main goals, the background and the motivation for the development of this dissertation.

Chapter 2 includes a brief literature review about what are microalgae and cyanobacteria, their applications, the cultivation systems employed and the importance of biofilms on

microalgal/cyanobacterial harvesting. Also, it focuses on reactors using microalgal biofilms for wastewater treatment.

Chapter 3 evaluates the surface physicochemical properties of the microalgae *C. vulgaris* and *P. subcapitata* and the cyanobacteria *S. salina* and *M. aeruginosa*, as well as those of selected materials (PS, SS 316, PMMA, G, PVC and Cu). A thermodynamic approach, predicting microbial adhesion to the studied materials is also provided.

Chapter 4 provides the study of the *in vitro* adhesion assays of the selected microorganisms to the previously referred materials. The effect of media composition on microalgal adhesion was also assessed. Also, the relationship between surface physicochemical properties and microalgal and cyanobacterial cells' adhesion was evaluated, making a connection between data obtained in Chapter 3 and Chapter 4.

Chapter 5 presents the nutrients removal from the culture media of two different culturing systems (STR and RDR) using a *C. vulgaris* biofilm. The optical density (OD) profile, as well as pH, temperature, light intensity and dissolved oxygen concentration were also evaluated.

Chapter 6 describes the main conclusions from this work and provides some proposals for future work.

CHAPTER 2

2. Literature review

2.1. Microalgae and cyanobacteria

Microalgae and cyanobacteria are a diverse group of prokaryotic and eukaryotic photosynthetic microorganisms that can grow rapidly under hard conditions, which make them the most abundant organisms on Earth [8, 9]. They are present in both marine and freshwater environments, representing a huge variety of species [10]. It is estimated that at least 50000 species exist, but only a small amount have been studied [11]. Moreover, it is estimated that the biomass productivity of microalgae and cyanobacteria could be much higher than that of other microorganisms, such as switchgrass [12].

These organisms can be classified into different phytoplankton taxonomic groups due to the wide range of structures, forms and sizes already reported [13]. They can be autotrophic, requiring inorganic compounds as carbon source and light as energy source or, otherwise, heterotrophic, requiring organic compounds as carbon and energy sources [14]. These microorganisms can also be mixotrophic, requiring both CO_2 and organic carbon (in this case, photosynthetic and respiratory metabolism occur simultaneously) [2, 15]. From now on, when we refer to microalgae, we include not only microalgae but also cyanobacteria.

2.2. Applications of microalgae and cyanobacteria

Over the past few years, the potential of microalgae and cyanobacteria in a wide range of applications has been ascertained, making them a valuable resource. Table 2.1 presents many applications already described for different microalgal/cyanobacterial species and the valuable products obtained by culturing these photosynthetic microorganisms.

Table 2.1. By-products and application areas described for different microalgal and cyanobacterial species

Microorganisms	Applications	By-products	References
Chlorella sp.	Human nutrition, cosmetics, wastewater treatment		[16, 17]
Chlorella minutissima	Food additive	Eicosapentaenoic acid	[8]
Spirulina sp.	Human and animal nutrition, cosmetics	Nutraceuticals, phycobiliproteins, γ-linolenic acid	[16, 18, 19]
Dunaliella salina	Human and animal nutrition, cosmetics, health food, food additive	Carotenoids	[14, 20-22]
Haematococcus pluvialis	Human and animal nutrition, cosmetics, aquaculture	Carotenoids, astaxanthin, leutin	[16]
Porphyridium sp.	Clinical immunology	Phycobiliproteins	[16, 20, 23]
Spirulina platensis	Food additive	Proteins, polyunsatured fatty acids, pigments, vitamins, phenolic compounds	[24-28]
Schizochytrium sp.	Food additive, nutraceuticals	Docosahexaenoic acid	[8]
Parietochloris incisa	Food additive, nutraceuticals	Arachidonic acid	[8]
Euglena gracilis	Human nutrition	Biotin, α-tocopherol (Vitamin E)	[8]
Prototheca moriformis	Human nutrition	Ascorbic acid (Vitamin C)	[8]
Isochrysis galbana	Animal nutrition	Fatty acids	[29, 30]
Phaeodactylum tricornutum	Human nutrition, biofuels	Fatty acids	[31-33]
Odontella aurita	Pharmaceuticals, cosmetics, baby food	Fatty acids	[21, 32]
Arthrospira sp.	Human and animal nutrition, cosmetics	Phycobiliproteins	[34-37]
Porphyridium cruentum	Health food, pharmaceuticals, cosmetics	Arachidonic acid, phycocyanin, phycoerythrin, extracellular polysaccharides	[38, 39]
Chlorella vulgaris	Wastewater treatment, health food, food additive, feed surrogates	Fatty acids	[2, 19]
Aphanizomenon flos-aquae	Human nutrition		[16]
Crypthecodinium cohnii	Food additive, wastewater treatment	Docosahexaenoic acid	[40]
Schizochytrium sp.	Food additive, wastewater treatment	Docosahexaenoic acid	[16]
Lyngbya majuscula	Pharmaceuticals, human nutrition	Immune modulators	[23]
Muriellopsis sp.	Health food, food additive, animal nutrition	Carotenoids, lutein	[21, 41]

2.3. Microalgal/cyanobacterial cultivation

Microalgae and cyanobacteria can be cultured in closed systems, also called photobioreactors (PBRs), or in open systems [42]. The viability of each system depends on several factors,

such as climatic conditions and intrinsic properties of the selected microalgal/cyanobacterial strain [14].

In PBRs, the cultures are totally enclosed within a vessel, enables the control of all essential parameters and, therefore, the reproducibility of the cultivation conditions [43, 44]. The most commonly used PBRs are the tubular, flat plate, and column ones [14, 45-47]. The use of closed PBRs is appropriated to overcome some problems related to the open pond systems [14]. Using these systems can be advantageous, since they have better light penetration, allowing higher biomass productivities and lower retention times. Also, PBRs allow the culture of single-species of microalgae and/or cyanobacteria for longer periods of time with less contamination risks and no CO_2 losses [44]. However, due to their complexity, they require more energy and specialised people. Therefore, the investment and operation costs are much higher than those required by open ponds [46, 48, 49]. The scale-up of this kind of systems is also more difficult [47].

On the other hand, on open pond systems the cultures are directly exposed to the environment, which makes them vulnerable to contamination by other microorganisms [43]. Shallow big ponds, circular ponds, raceway ponds and tanks are the most commonly used systems [19, 46]. Some conditions, such as temperature and light, cannot be controlled for optimal microalgal/cyanobacterial growth. Therefore, the outdoor cultivation for commercial use is highly restricted to warm tropical locations, with low precipitation and cloud cover [50]. Although they have larger production capacities, biomass productivities are lower than those achieved in PBRs, since they have poor mixing and oscillations in the culture conditions are very common [44-46, 51]. However, these systems are simpler than PBRs and, consequently, less expensive in terms of operation and production costs [52]. The high evaporation rates can be seen as a disadvantage, but it also brings benefits to the culture system, since it helps with temperature control thanks to evaporative cooling [51].

Both PBRs and open ponds are technologies based on microalgal/cyanobacterial growth in suspension. Indeed, the existing systems for both biomass production and wastewater treatment focus on microalgal/cyanobacterial growth in suspension on the culture medium. However, as biomass removal is essential for water recycling and commonly used harvesting procedures present some economic and technical limitations, new alternatives should be

developed to optimize microalgal/cyanobacterial recovery. In this sense, microalgal/cyanobacterial immobilization has appeared as a valuable alternative for planktonic cultivation systems, since the cultured microorganisms grow in an immobilized form, making easy their removal at the end of cultivation time. Additionally, it has been reported that the application of an immobilized system in wastewater treatment processes allows more flexibility in terms of reactor design, comparatively to the planktonic ones [53]. Besides, immobilization provides higher cell densities, increased cell wall permeability and prevents washout and low leakage of cells from the matrix [53-55]. The immobilization procedure begins with cell growth in a reactor, followed by harvesting and immobilization at a very high cell density. In 1966, for the first time in history, a study on immobilized algae was performed [56]. However, Prof. de la Noüe and his co-workers were the pioneers in using this technique applied to wastewater treatment. The promising results obtained have generated an increase in reports about microalgal/cyanobacterial immobilization for wastewater remediation applications [53].

There are many methods of immobilization, such as adsorption, crosslinking, entrapment, covalent bonding and encapsulation. Additionally, immobilization techniques comprise the immobilization in the surface of a substrate, in the form of biofilm, and the immobilization on the core of a matrix. An ideal substrate or matrix for immobilization should present the following properties: non-toxicity, stability, biomass retention, phototransparency, as well as resistance to disruption by cell growth [53]. These can be either a natural polymer, such as agar, agarose, collagen, cellulose, carrageen and alginate, or a synthetic polymer, such as polyurethane, polyvinyl chloride and acrylamide [57]. A summary of the substrates and matrixes described for microalgal/cyanobacterial immobilization systems is presented in Table 2.2.

Table 2.2. Summary of microalgae successfully immobilized onto different substrates and matrixes

Microorganisms	Substrates/Matrixes	Reference
Chlorella vulgaris	Glass; 3-aminopropyltriethoxysilane*; Propyltriethoxysilane*	[58]
Scenedesmus obliquus *Chlorella vulgaris* *Coccomyxa* sp. *Nannochloris* sp. *Nitzschia palea* *Oocystis* sp. *Oocystis polymorpha*	Polystyrene; Polycarbonate; Acrylic; Glass	[59]
Chlorella vulgaris *Nitzschia amphibia* *Chroococcus minutus*	Poly(methyl methacrylate); Titanium; Glass; Copper**; Stainless steel; Admiralty brass**; Aluminum brass**	[60]
S*cenedesmus obliquus* *Chlorella vulgaris*	Boorosilicate glass; Polyethylene; Polyurethane; Poly(methyl methacrylate)	[61]
Botryococcus braunii *Botryococcus sudeticus* *Ankistrodesmus falcatus* *Cylindrotheca fusiformis* *Amphora coffeaeformis* *Nitzschia frustulum* *Scenedesmus dimorphus* *Chlorella vulgaris* *Nannochloropsis oculata*	Glass; Indium-tin oxide; Stainless steel; Polycarbonate; Polyethylene; Polystyrene	[62]
Prototheca zopfii	Polyurethane	[63]
Aulosira fertilissima	Glass	[64]
Scenedesmus acutus *Chlorella vulgaris*	Polyurethane	[65]
Phormidium laminosum *Phormidium uncinatum*	Polyvinyl chloride	[66, 67]
Chlorella kessleri *Chlorella vulgaris*	Polystyrene Polyurethane	[68]
Scenedesmus obliquus *Chlorella vulgaris* *Coccomyxa* sp. *Nannochloris* sp. *Nitzschia palea* *Oocystis* sp. *Oocystis polymorpha*	Cellulose acetate; Silicone rubber	[59]
Ascophyllum nodosum *Chlorella vulgaris* *Anabaena doliolum* *Chlorella emersonii* *Chlorella homoshaera* *Chlorella salina* *Chlorella ellipsoidea* *Scenedesmus quadricauda* *Navicula canalis* *Nannochloropsis gaditana* *Scenedesmus obliquus* *Tetraselmis chuii*	Alginate	[69-80]
Chlorella emersonii	Agarose	[77]

* Modified surfaces; ** Poor attachment.

Table 2.2. (Continued)

Microorganisms	Substrates/Matrixes	Reference
Spirulina platensis *Chlorella vulgaris*	Silica gel	[81]
Scenedesmus acutus *Chlorella vulgaris*	Carrageenan	[65]
Chlorella sorokiniana	Loofa sponge	[82]
Phormidium sp. *Scenedesmus bicellularis*	Chitosan	[83, 84]
Phormidium laminosum	Cellulose	[85]
Chlorella kessleri *Chlorella vulgaris*	Carrageenan; Alginate	[68]
Nostoc muscorum *Mastigocladus laminosus*	Agar	[86]

* Modified surfaces; ** Poor attachment.

2.4. Microalgal/cyanobacterial biofilms

A biofilm can be designed as a layer of cells attached to a substrate and embedded in an organic and biological matrix [7]. Microalgal biofilms are dominated by microalgae or cyanobacteria (which we include as microalgae) [87]. However, some other microorganisms can be presented in microalgal biofilms, mainly bacteria, which have demonstrated to favour biofilm formation [88]. Microalgal biofilms have the ability to adapt to different environmental conditions, to detach from the substrate as a single colony or in clumps, and to support cells [89]. Due to their structure complexity and community dynamics it is difficult to accurately reproduce biofilms within a laboratory [90]. Biofilms can be either beneficial or injurious, depending on which area they are applied. In industry, biofilms reduce the heat transfer and are responsible for fouling membranes and contamination of food process equipment. In the wastewater treatment field, they are essential and play a key role in biomass harvesting, nutrients level reduction and operation costs reduction [7]. The process of biofilm formation is characterized by five stages, as it can be observed in Figure 2.1. In stage 1 there is an initial attachment of cells to the surface, followed by the production of the extracellular matrix, consisting of extracellular polymeric substances (EPS) and deoxyribonucleic acid, which results in more firmly adhered cells: “irreversible” attachment (stage 2). In stage 3, a vertical development of the biofilm architecture occurs. Then, the biofilm architecture grows (stage 4) and, finally, when the biofilm is large enough, some areas of the extracellular matrix are degraded by enzymes, leading to the dispersion of cells from the biofilm that can begin another process of biofilm formation [91].

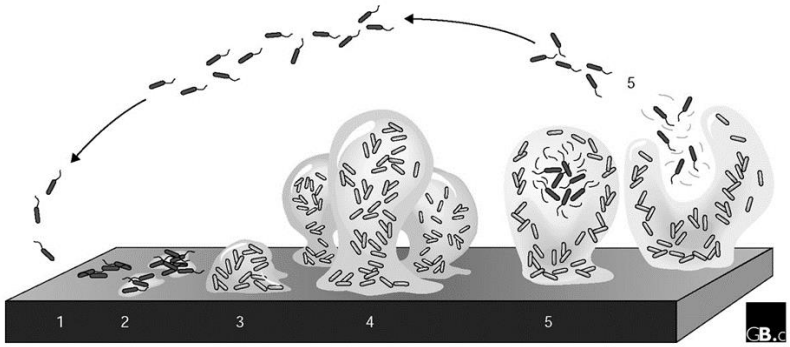

Figure 2.1. Phases of algal biofilms formation: 1 - initial attachment; 2 - attachment; 3 - 1st maturation phase; 4 - 2nd maturation phase; 5 - detachment. Adapted from Stoodley et al. [91].

Biofilms may be formed in a wide variety of surfaces and conditions [92]. Due to environmental factors, microorganisms can change from the planktonic form to the sessile form [93]. There are many factors influencing microalgal/cyanobacterial biofilm formation, such as environmental conditions (light, pH, nutrient composition, temperature), hydrodynamic conditions, the properties of the adhesion surface (hydrophobicity) and the characteristics of the microorganism (motility, surface hydrophobicity, chemotaxis, metabolic interactions, cell size, ability to produce EPS) [93, 94].

Light is one of the most important environmental factors that influences the development of microalgal/cyanobacterial biofilms [95]. Light can affect the formation of algal biofilms through both photoinhibiton (excessive light supply) or photolimitation (low light supply) across the biofilm upper layers [61, 89]. Many researchers have found that photon flux densities can be critical in microalgal biofilm growth [80, 96, 97], but the specific value at which photoinhibition or light limitation occur varies with species [89]. Although light intensity is critical for microalgal biofilm growth, light quality is also extremely important as it can affect lipid composition and content and, therefore, biofilm growth [98-100]. Additionally, light affects nutrients removal by influencing microalgal growth, whereby the selection of appropriate light conditions is essential for successful application of microalgal and cyanobacterial biofilms in wastewater treatment processes [89].

During microalgal/cyanobacterial biofilm growth, the concentration of CO_2 is affected by the pH, and the pH is affected by the utilization of CO_2. More specifically, since photosynthesis requires CO_2, there is a rapid consumption of this compound, thus raising the pH of the culture medium. As CO_2 becomes limiting with distance into the biofilm due to the total

inorganic carbon decrease, the HCO_3^- will form CO_2 and OH^-, leading to pH increase and the establishment of an equilibrium [101, 102].

Nutrients limitation can affect the biofilm growth, the type of biofilm produced and the composition of species present on the biofilm [89, 103, 104]. Heterotrophic biofilms can be favoured by high contents of biodegradable organic matter [105]. Conversely, photoautotrophic biofilms can be enhanced by the presence of inorganic nutrients and light [106]. Carbon, nitrogen and phosphorus are the most important nutrients for biofilm growth. So, C:N:P ratios are widely used to predict and minimize nutrient limitation in microalgae and cyanobacteria growth [107, 108].

Many metabolic reactions are influenced by temperature, meaning that microalgal/cyanobacterial biofilm formation is also affected by temperature oscillations [109]. Some authors have found that warm temperatures can promote biofilm formation, whereas lower temperatures can inhibit biofilm growth [89, 109, 110]. So, it is expected that in tropical regions, where temperature is usually warm, biofilm formation is higher than in cold regions [109]. It has been reported that diatoms are established at temperatures from 5 to 15 °C, green algae at 15 to 30 °C, and cyanobacteria above 30 °C [111]. However, the biofilm response to temperature changes is known to be species-dependent [89].

The type of surface used can also influence biofilm formation. The most important surface properties are hydrophobicity, surface tension, roughness and charge. Several studies have demonstrated that the extent of biofilm increases as the surface roughness increases, since the increased area provides more sites for colonization [112-114]. According to the literature [115, 116], a small increase in surface roughness leads to a significant adhesion of microbial cells. However, a larger increase in surface roughness can have no significant effect in microbial adhesion. Also, porous surfaces have been associated to higher cell attachment [117]. Regarding hydrophobicity, some authors have demonstrated that cells adhere more to surfaces with low free energy of hydrophobic interaction (hydrophobic surfaces) than to hydrophilic surfaces [118-120].

Biofilms can grow in laminar or turbulent flow [113]. Hydrodynamic conditions control drag forces and mass transfer (transport of cells, oxygen and nutrients), playing an important role on biofilm formation [121, 122]. Biofilms developed under laminar flow are different from

those of turbulent flow. The first ones correspond to cell aggregates with interstitial voids, whereas the others consist of elongated streamers oscillating in the bulk fluid and forming a thicker biofilm [113, 123]. Therefore, the hydrodynamic conditions imposed into the system will influence the nature of the biofilm, as well as the phenotype of microbial cells.

All benthic algae, diatoms and cyanobacteria produce EPS [124]. Diatoms are the most abundant EPS producers [125]. The attachment of cells and biofilm formation is not possible without a matrix of EPS. EPS consist of a wide diversity of organic molecules, such as proteins, polysaccharides, amino-acids and uronic acid [109, 126]. They have many advantages, such as protection of microorganisms from toxic substances and prevention of cells from desiccation [109]. These substances are responsible for biofilms mechanical stability and cohesion and mediate their attachment to surfaces [125]. Also, some authors found that a coating of EPS can protect microalgae and cyanobacteria against phagocytosis, dehydration, lysis, antibody recognition and contact with toxic heavy metals [127-132]. EPS represent 50-90% of the organic carbon of the biofilm [133]. Both uronic acids and sulphate groups contribute to the negative charge (anionic nature) of the EPS, explaining the affinity between EPS and cations, such as metal ions. So, the presence of negatively charged polysaccharides around microalgae and cyanobacteria may help in metal cations sequestration, which can be favourable when there are low concentrations of metals that are essential for biofilm growth. [134].

2.5. The role of microalgal/cyanobacterial biofilms in wastewater treatment processes

The recent increase of industrialization, agricultural practices and urbanization have conducted to an excessive input of nutrients in water resources [2]. Worldwide, water pollution has become a major problem as it can lead to the degradation of natural ecosystems. Therefore, there is an urgent need for new wastewater treatment technologies that can effectively remove nutrients and, at the same time, be economically feasible [135]. In fact, wastewaters are mainly composed by water (99.9%) and organic and inorganic compounds (0.1%) [136]. Among all nutrients present in wastewater, nitrogen and phosphorus are the most abundant and, when in excess, can cause eutrophication [137]. Wastewaters may also contain heavy metals, such as cadmium, copper, zinc and lead, which can be dangerous for human health if not removed [137]. Recently, with the increasing concerns about wastewater

remediation strategies for nutrient control, microalgal and cyanobacterial biofilms have emerged as a cost-effective and eco-friendly alternative to microalgal suspended-based technologies [89, 138]. The microalgal/cyanobacterial remediation mechanism relies on nitrogen and phosphorus uptake from the effluent and the assimilation of these inorganic nutrients towards their growth [139] Besides, biofilms have the ability to retain biomass that, after chemical and biological processing, can be transformed into high valued products and fertilizers [4, 140], resulting in the recycling of the nitrogen and phosphorus wastes. Their ability to retain microalgal/cyanobacterial biomass in a substrate or material, makes microalgal biofilms a sustainable alternative for wastewater treatment, reducing the costs associated to the harvesting of biomass before effluent discharge into water bodies [4, 141].

Table 2.3. Microalgal/Cyanobacterial biofilm reactors, operation conditions and nutrients removal efficiencies reported in the literature for the treatment of wastewaters from different sources

Microorganisms	Reactor	Conditions	Wastewater source	Nutrients removal	Reference
Chlorella spp.+ *Nitzchia* spp.	ND	OM=ND; V=15 L; HRT=10 h; T=ND; LI=400 ft.c; L:D=ND	Settled domestic sewage	92% N 74 % P	[142]
Microalgal consortia	Flow cell	OM=continuous; V=0.36 L; HRT=ND; T=22ºC; LI=230 µmol m^{-2} s^{-1}; L:D=ND	Domestic wastewater	ND	[4]
Chlorella vulgaris	Two raceways	OM=continuous; V=2200 L; HRT=4.5 d; T=ND; LI=ND; L:D=ND	Diluted pig slurry	54-98% N 42-89% P	[143]
Chlorella vulgaris	Algal biofilm membrane photobioreactor	OM=continuous; V=ND; HRT=2 d; T=25-28 ºC; LI=8000 lux; L:D=ND	Simulated secondary effluent	96% N 85% P	[144]
Scenedesmus sp.	PMMA reactor with cylindrical polypropylene fiber bundle carriers	OM=continuous; V=96 L; HRT=2 d; T=20-22 ºC; LI=2800 lx; L:D=6:6	Municipal wastewater	24-55% N	[145]
Microalgal consortia	Lab scale ATS raceways with polyethylene mesh	OM=ND; V=3700 L; HRT=ND; T=19-24ºC; LI=270-390 µmol m^{-2} s^{-1}; L:D=23:1	Anaerobically digested dairy manure	70-90% N 70-90% P	[146]
S. obliquus *C. vulgaris*	PMMA flow cell with coupons	OM=fed-batch; V=0.48 mL; HRT=ND; T=23-27 ºC; LI=100 µmol m^{-2} s^{-1}; L:D=24:0	Municipal wastewater	ND	[147]

PMMA - poly(methyl methacrylate); ATS - algal turf scrubber; RABR - rotating algal biofilm reactor; OM - operation mode; V - volume; HRT - hydraulic retention time; T - temperature; LI - light intensity; L:D - light:dark photoperiod; ND - not defined; N - nitrogen; P - phosphorus.

Table 2.3. (Continued)

Microorganisms	Reactor	Conditions	Wastewater source	Nutrients removal	Reference
Trentepohlia aurea	Filter paper	OM=ND; V=ND; HRT=ND; T=25 ºC; LI=10 µmol m^{-2} s^{-1}; L:D=24:0	Synthetic wastewater	75% N	[148]
Microalgal consortia	Polycarbonate flow lanes photobioreactor	OM=ND; V=ND; HRT=ND; T=20-30 ºC; LI=15-120 µmol m^{-2} s^{-1}; L:D=16:8	Domestic sewage	100% P	[149]
Chlorella sp.	Polystyrene rocker system	OM=batch; V=ND; HRT=6 d; T=20 ºC; LI=110-120 µmol m^{-2} s^{-1}; L:D=24:0	Dairy manure	62% N 73% P	[150]
Microalgal consortia	Lab scale ATS units with polyethylene mesh	OM=continuous; V=ND; HRT=ND; T=22 C; LI=40-140 µmol m^{-2} s^{-1}; L:D=16:8	Raw and anaerobically digested dairy manure	98% N 99% P	[151]
Microalgal consortia	Pilot scale RABR	OM=continuous; V=8000 L; HRT=0.25 d; T=9.6-19.2 ºC; LI=170 µmol m^{-2} s^{-1}; L:D=14:10	Municipal wastewater	76% N 23.8% P	[138]
Chlorella sorokiniana Ralstonia basilensis	Flate plate photobioreactor	OM=continuous; V=0.709 L; HRT=3 d; T=25ºC; LI=180 µE m^{-2} s^{-1}; L:D=14:10	Mineral salt medium	ND	[152]
Chlorella sorokiniana Ralstonia basilensis	Algal turf reactor shallow open pond	OM=continuous; V=0.13 L; HRT=1.3 d; T=25 ºC; LI=180 µE m^{-2} s^{-1}; L:D=14:10	Mineral salt medium	ND	[152]
Chlorella sorokiniana Ralstonia basilensis	Tubular photobioreactor	OM=continuous; V=0.251 L; HRT=2.3 d; T=25 ºC; LI=180 µE m^{-2} s^{-1}; L:D=14:10	Mineral salt medium	ND	[152]

PMMA - poly(methyl methacrylate); ATS - algal turf scrubber; RABR - rotating algal biofilm reactor; OM - operation mode; V - volume; HRT - hydraulic retention time; T - temperature; LI - light intensity; L:D - light:dark photoperiod; ND - not defined; N - nitrogen; P - phosphorus.

Several studies have focused on nutrients removal from wastewaters using microalgal and cyanobacterial biofilms [153-155]. Among all microalgae applied in wastewater treatment for nutrients removal, *Chlorella*, *Spirulina*, *Phormidium*, *Botryococcus*, *Chlamydomonas* and *Scenedesmus* are the most widely used genera [2, 156]. The reactors/technologies used on some of those studies are described in Table 2.3, along with the operational conditions applied and nutrients removal efficiencies achieved.

The studies summarized in Table 2.3 show that microalgal and cyanobacterial biofilms systems can be used to effectively remove nitrogen and phosphorus from wastewaters.

However, their efficacy will rely on many other factors apart from nutrients removal efficiencies, such as the biomass productivities, the area required and the final concentration of wastewater, that will have to be in accordance with the limits for discharge effluents defined by European Union (EU) legislation [157].

CHAPTER 3

3. Understanding microalgal and cyanobacterial adhesion through characterization of surface physicochemical properties

3.1. Introduction

Microalgae have a wide range of applications, going from food industry to cosmetics [8, 16, 18, 20]. For many years, microalgae have been used in wastewater treatment. However, for the past few years, the interest in microalgae has largely increased, since some findings suggested that when wastewater is used as nutrient supply, the biofuel production by these microorganisms can be economically feasible and environmentally friendly [139, 158, 159]. Besides, the use of microalgae in wastewater treatment processes have many other advantages, such as low operational costs, no organic carbon requirement, decreasing of CO_2 emissions associated to wastewater treatment plants, the obtainment of an oxygenated effluent, heavy metals removal in a safer way, recycling of phosphorus and nitrogen into microalgal biomass, which can be used as a fertilizer, and reduction in sludge formation [2, 57, 160-162].

Microalgae can be used in different stages of the wastewater treatment processes: (i) after a load sludge system, as a post-treatment system (tertiary treatment phase); and (ii) in combination with bacteria in the secondary treatment phase [163]. Among all microalgal species applied in wastewater treatment for nutrients removal, *Chlorella*, *Spirulina*, *Phormidium*, *Botryococcus*, *Chlamydomonas* and *Scenedesmus* are the most widely used genera [2, 156]. Several studies have been conducted using single cultures of microalgae or cyanobacteria, but the combination of more than a single microorganism has shown to be more advantageous than single cultures [164-166].

However, the major drawback associated to wastewater treatment using microalgae is the separation of the produced biomass from the treated effluent [1, 161]. The methods commonly used for microalgal harvesting are chemical flocculation, bioflocculation, gravitational sedimentation, filtration, electrocoagulation-flocculation, flotation, centrifugation and the combination of any of these [164, 167]. Among all the existing methods, immobilization systems seem to be a good alternative, since the microorganisms grow in an immobilized form, making easier their recovery from the effluent. Therefore, microalgal biofilms have gained particular attention in wastewater treatment systems, as they can promote a rapid and efficient harvesting of biomass [1].

Cell adhesion to a surface is a step that precedes biofilm formation. It is well established that the type of microorganism, light intensity, temperature, pH, media composition, hydrodynamic conditions, EPS production and quorum sensing phenomena are key factors influencing the attachment of microalgal/cyanobacterial cells and, consequently, biofilm development [93, 94, 168]. Nevertheless, many studies have focused on the effects of physicochemical properties of the surfaces on microbial adhesion and biofilm formation [169]. Although not always truth, it is reported in the literature that there is a trend of microorganisms to adhere to hydrophobic surfaces [170-173].

The aim of this chapter was to characterize the surface physiochemical properties of two microalgae, *C. vulgaris* and *P. subcapitata*, and two cyanobacteria, *S. salina* and *M. aeruginosa.* Some hydrophobic and hydrophilic surfaces were also characterized: polystyrene (PS), stainless steel 316 (SS 316), poly(methyl methacrylate) (PMMA), glass (G), polyvinyl chloride (PVC) and copper (Cu). Selection of these surfaces was based on the following factors: (i) SS 316 is widely used in industrial applications; (ii) PS, PMMA and G are transparent surfaces, which allow light penetration; (iii) PVC is a cheap and resistant material; and (iv) Cu serves as a control, since its cellular toxicity has already been reported in the literature [174-177]. The free energy of hydrophobic interaction and the free energy of adhesion between the studied microorganisms and surfaces were based on contact angles measurements, which helped to identify important forces involved on microalgal/cyanobacterial adhesion.

3.2. Materials and methods

3.2.1. Microorganisms and culturing conditions

The microalgae *C. vulgaris* CCAP 211/11B and *P. subcapitata* CCAP 278/4 were obtained from Culture Collection of Algae and Protozoa (United Kingdom), while the cyanobacteria *S. salina* LEGE 06079 and *M. aeruginosa* LEGE 91344 were obtained from the Laboratory of Ecotoxicology, Genomic and Evolution – CIIMAR (Centre of Marine and Environmental Research of the University of Porto, Portugal). Stock solutions of these microorganisms were prepared in OECD (Organization for Economic Co-operation and Development) test medium [178], with the following composition (per litre): 250 mg $NaNO_3$, 12 mg $MgCl_2 \cdot 6H_2O$, 18 mg $CaCl_2 \cdot 2H_2O$, 15 mg $MgSO_4 \cdot 7H_2O$, 45 mg KH_2PO_4, 0.08 mg $FeCl_3 \cdot 6H_2O$, 0.1 mg $Na_2EDTA \cdot 2H2O$, 0.185 mg H_3BO_3, 0.415 mg $MnCl_2 \cdot 4H_2O$, 3 μg $ZnCl_2$, 1.5 μg $CoCl_2 \cdot 6H_2O$, 0.01 μg $CuCl_2 \cdot 2H_2O$, 7 μg $Na_2MoO_4 \cdot 2H_2O$, and 50 mg $NaHCO_3$. Culture medium was sterilized by autoclaving at 121 °C for 15 min. The cells were incubated in 500-mL flasks at room temperature (24.0±1.0 °C), under continuous fluorescent light with an irradiance of approximately 120 $\mu E\ m^{-2}\ s^{-1}$ at the surface of the flasks. Agitation was obtained by bubbling atmospheric air (filtered through 0.22-μm cellulose acetate membranes, Orange Scientific, Belgium) in the bottom of the flasks.

3.2.2. Surface contact angle measurements

Microalgal suspensions in the exponential growth phase were harvested, washed twice and resuspended in saline solution (0.85% w/v, NaCl) to obtain a final concentration of about 5.0×10^6 cells mL^{-1}. Algal lawns were prepared by filtering the previously washed suspensions using 0.45-μm nitrocellulose membrane filters (ADVANTEC MFS, Inc., Japan) until complete clogging of the membranes. Contact angle measurements were performed using the sessile drop method, as described by Busscher, Weerkamp [179]. The measurements were carried out at room temperature (23±2 °C) using water, formamide and α-bromonaphthalene (Sigma-Aldrich, Portugal) as the reference liquids. Determination of contact angles was performed automatically using a model OCA 15 Plus (DATAPHYSICS, Germany) video based optical contact angle measuring instrument, allowing image acquisition and data analysis. Contact angle measurements (at least 12 determinations for

each liquid and for each algal suspension/material) were performed for two independent experiments.

3.2.3. Surface parameters and hydrophobicity determination

After contact angle measurements, values of surface hydrophobicity of the studied algal suspensions and materials were determined using the approach of van Oss [180], which allows the assessment of the absolute degree of hydrophobicity of any surface in comparison with their interaction with water. In this approach, the degree of hydrophobicity of a given surface (s) is expressed as the free energy of hydrophobic interaction between two entities of that surface when immersed in water (w): ΔG_{sws}^{TOT}, in mJ m^{-2}. When $\Delta G_{sws}^{TOT} < 0$, the interaction between the two entities is stronger than the interaction of each entity with water and the material is considered hydrophobic. Alternatively, if $\Delta G_{sws}^{TOT} > 0$, the material is hydrophilic. ΔG_{sws}^{TOT} can be calculated through the surface tension components of the interacting entities, according to Equation 3.1 [181-183]:

$$\Delta G_{sws}^{TOT} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_s^+\gamma_w^-} + \sqrt{\gamma_s^-\gamma_w^+} - \sqrt{\gamma_s^+\gamma_s^-} - \sqrt{\gamma_w^+\gamma_w^-}\right) \quad (3.1)$$

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

The surface tension components of a surface (s) were obtained by measuring the contact angles of three pure liquids (l), water and formamide (both polar) and α-bromonaphthalene (apolar), followed by the simultaneous resolution of three equations of the form of Equation 3.2. Surface tension of liquid components were obtained from the literature [184].

$$(1 + \cos\theta) \cdot \gamma_l^{TOT} = 2\left(\sqrt{\gamma_s^{LW}\gamma_l^{LW}} + \sqrt{\gamma_s^+\gamma_l^-} + \sqrt{\gamma_s^-\gamma_l^+}\right) \quad (3.2)$$

where θ is the surface-liquid contact angle and $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$. Contact angle of the apolar liquid, α-bromonaphthalene, was used to quantify the apolar energy component γ_s^{LW}, since γ_l^- and γ_l^+ for this probe liquid are equal to zero. On the other hand, contact angles

measured with the other probe liquids were used to determine the other surface parameters, γ_s^+ and γ_s^-.

3.2.4. Free energy of adhesion determinations

The free energy of adhesion between the studied microalgae and surfaces was performed according to Simões et al. [185]. When studying the interaction between surface *i* (microalgal/cyanobacterial cells) and *I* (SS 316, PS, PMMA, G, PVC and Cu) that are immersed or dissolved in water, the total interaction energy ($\Delta G_{adhesion}$, in mJ m^{-2}) can be assessed through the following expression:

$$\Delta G_{adhesion} = \gamma_{iI}^{LW} - \gamma_{iw}^{LW} - \gamma_{Iw}^{LW} + 2\left[\sqrt{\gamma_w^+}\left(\sqrt{\gamma_i^-} + \sqrt{\gamma_I^-} - \sqrt{\gamma_w^-}\right) + \sqrt{\gamma_w^-}\right] \times \left(\sqrt{\gamma_i^+} + \sqrt{\gamma_I^+} - \sqrt{\gamma_w^+}\right) - \sqrt{\gamma_i^+\gamma_I^-} - \sqrt{\gamma_i^-\gamma_I^+} \quad (3.3)$$

Thermodynamically, adhesion is expected to occur if $\Delta G_{adhesion} < 0$, while if $\Delta G_{adhesion} > 0$, microbial adhesion is not favoured.

3.2.5. Statistical analysis

Contact angles, surface tension parameters, free energy of hydrophobic interaction and free energy of adhesion values were analysed using paired-samples *t*-test from the statistical software SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Statistical calculations were based on a confidence level ≥95% (p<0.05 was considered statistically significant).

3.3. Results and discussion

3.3.1. Surface physicochemical properties of the selected microorganisms

The contact angles measurements allowed the determination of the surface tension parameters for *C. vulgaris*, *P. subcapitata*, *S. salina* and *M. aeruginosa*. These results are presented in Table 3.1, in the decreasing order of free energy of hydrophobic interaction (from the most hydrophilic to the most hydrophobic suspensions).

From Table 3.1, it is possible to conclude that all cell surfaces were hydrophilic, since $\Delta G_{sws}^{TOT} > 0$ mJ m^{-2}. Therefore, it is expected that the cells may be stable in the dispersed form

and will not form aggregates. Gonçalves et al. [164] obtained similar results of ΔG_{sws}^{TOT} for *C. vulgaris, P. subcapitata* and *M. aeruginosa* (52.5, 13.8 and 43.4 mJ m^{-2}, respectively). Similarly, Ozkan and Berberoglu [186] studied the physicochemical properties of *Synechocystis* sp. and verified a hydrophilic character (ΔG_{sws}^{TOT} of 10.9 mJ m^{-2}) for this cyanobacteria, which corroborates the data obtained in this study for *S. salina.*

The Lifshitz van der Waals component, γ_s^{LW}, was similar for all studied microorganisms assessed, with values ranging from 36.3±0.6 to 38.6±1.4 mJ m^{-2} (p>0.05). Several studies have obtained similar results, with values between 35.0 and 37.8 mJ m^{-2} [164, 186]. LW forces, usually attractive, result from instantaneous asymmetrical distribution of electrons in molecules (the higher the value of LW component, the more apolar is the surface and, therefore, the lower would be its affinity for polar liquids) [62]. Similar results obtained for this component indicate that cell wall composition of the studied microorganisms may not have significant differences.

Electron donor and acceptor parameters give information about the molecules present in the studied surface: higher γ_s^+ indicate the presence of positively charged molecules and higher γ_s^- indicate the presence of negatively charged ones [187]. In this study, all microorganisms presented electron donor qualities, with a predominance of the electron donor component, γ_s^-, which may result from the presence of excessive molecules of oxygen on the surface of the microorganisms and the neutralization of the few γ_s^+ sites by the dominant γ_s^- sites, through intra- and intermolecular interactions [164, 187].

Regarding the acid-base or polar component, values of γ_s^{AB} determined for the studied microalgae ranged from 5.8±1.0 to 12.7±16.1 mJ m^{-2}, whereas the same values determined for the cyanobacteria ranged from 6.3±8.3 to 7.9±6.3 mJ m^{-2}. This component was not statistically different (p>0.05) among the studied microorganisms. In the study performed by Ozkan and Berberoglu [186], similar values of γ_s^{AB} were obtained for green algae, ranging from 0.0 to 5.1 mJ m^{-2}. Acid-base forces result from electron transfer interactions between polar components of the involved surfaces. These interactions can be attractive (hydrophobic attraction) or repulsive (hydrophilic repulsion), depending on the free energy of hydrophobic interaction. Since the studied microorganisms presented hydrophilic surfaces, γ_s^{AB} values determined are a measure of the hydrophilic repulsion.

Table 3.1. Surface tension parameters and hydrophobicity of the selected microorganisms and materials. The values are presented as the mean±SD of two independent experiments

	Surface tension parameters (mJ m^{-2})				Hydrophobicity	Contact angle (°)		
Surface	γ_s^{LW}	γ_s^+	γ_s^-	γ_s^{AB}	ΔG_{sws}^{TOT}	θ_w	θ_B	θ_F
M. aeruginosa	37.8±5.5	0.4±0.5	49.3±6.5	6.3±8.3	31.2±6.7	34.5±8.7	31.1±14.9	38.6±4.7
S. salina	36.5±0.6	0.5±0.7	46.4±10.7	7.9±6.3	28.0±17.5	36.6±4.0	35.5±1.4	33.5±0.1
P. subcapitata	38.6±1.4	0.2±0.1	43.0±1.3	5.8±1.0	22.9±0.7	39.9±3.4	30.0±3.8	39.1±4.1
C. vulgaris	36.3±0.6	0.3±0.4	48.4±5.7	12.7±16.3	18.1±30.5	37.4±0.2	36.0±1.4	41.3±6.1
SS 316	37.4±4.2	1.0±1.5	42.2±16.7	10.6±15.0	17.0±18.8	34.4±29.2	32.7±11.1	33.2±23.5
PMMA	43.2±0.2	0.0±0.0	34.5±12.3	0.0±0.0	8.5±21.3	54.7±9.1	13.5±8.2	53.3±2.0
G	34.2±00*	3.7±0.0*	14.8±0.0*	14.9±0.0*	-17.8*±0.0	56.5±0.0*	40.9±0.0*	28.4±0.0*
PS	38.4±0.0*	0.0±0.0*	19.3±0.0*	0.0±0.0*	-18.0±0.0*	93.1±0.0*	30.6±0.0*	98.7±0.0*
PVC	40.7±0.6	0.1±0.2	18.9±1.6	2.1±3.0	-19.3±2.9	66.5±6.8	23.9±1.8	55.0±14.2
Cu	40.7±0.4	0.0±0.0	5.6±3.3	0.0±0.0	-61.4±14.4	94.2±2.1	23.7±1.4	80.5±3.6

* This value is related to one replicate only. Additional replicates should be performed to confirm these results.

γ_s^{LW} - Lifshitz van der Waals component of the surface tension (mJ m^{-2}); γ_s^+ - electron acceptor component of the surface tension (mJ m^{-2}); γ_s^- - electron donor component of the surface tension (mJ m^{-2}); γ_s^{AB} - acid-base component of the surface tension (mJ m^{-2}); ΔG_{sws}^{TOT} - free energy of hydrophobic interaction (mJ m^{-2}); θ_W - contact angle using water (°); θ_B - contact angle using α-bromonaphthalene (°);θ_F - contact angle using formamide (°).

3.3.2. Surface physicochemical properties of the selected materials

The surface tension parameters were also determined for the six studied materials. As for microbial surfaces, these results are presented in Table 3.1 in the decreasing order of free energy of hydrophobic interaction (from the most hydrophilic to the most hydrophobic materials). As it can be observed from Table 3.1, contact angles determined using the polar liquids present values below 90º, with exception of PS and Cu, meaning that surface-liquid interactions dominate the system. Both PMMA and SS 316 were hydrophilic ($\Delta \boldsymbol{G}_{sws}^{TOT} >$ 0 mJ m^{-2}). As stated in the literature, surfaces presenting higher free energy of hydrophobic interaction are more hydrophilic, which is the case of SS 316 [113, 188]. Although the results showed a hydrophilic character of PMMA, several studies have demonstrated the opposite [118, 188, 189]. This contradiction may result from different nature, finishing or cleaning treatment of the material [190].In contrast, PS, PVC, G and Cu are hydrophobic surfaces, due to the negative value of $\Delta \boldsymbol{G}_{sws}^{TOT}$. Some authors [190-192] obtained similar values for G (-13.8 mJ m^{-2}) and Cu (-79.6 mJ m^{-2}). However, it was found that the free energy of hydrophobic interaction values determined for PVC and PS were smaller than those reported in the literature, being -55.9 and -44.0 mJ m^{-2} for PVC and PS, respectively [190]. These differences may also occur due to different nature, finishing or cleaning treatment of the material.

The γ_s^{LW} value was almost the same (p>0.05) for all surfaces, ranging from 34.2 (G) to 40.7 mJ m^{-2} (PMMA and Cu).

Comparing electron acceptor (γ_s^+**)** and donor (γ_s^-**)** parameters, it is possible to conclude that all surfaces are more predisposed to donate electrons than to accept them. Data obtained for γ_s^+ and γ_s^- between the studied surfaces was not statistically different (p>0.05). Several studies [181, 182, 193] predicted the nonexistence of electron acceptor parameters, which corroborate the γ_s^+ values close to zero obtained in this study.

Looking at γ_s^{AB} parameter, the values determined for the studied materials ranged from 0.0±0.0 to 14.9±0.0 mJ m^{-2}. These values were not statistically different (p>0.05), except in the case of PVC and G, where statistically higher (p<0.05) values were obtained. In the case of SS 316 and PMMA, determined values of γ_s^{AB} are a measure of hydrophilic repulsion,

whereas in the case of PS, PVC, G and Cu, these values are a measure of hydrophobic attraction.

3.3.3. Free energy of adhesion

In order to predict the ability of the microorganisms to adhere to the studied materials, the free energy of interaction between the microalgae/cyanobacteria and the surface when immersed in water, also known as free energy of adhesion, was calculated according to the thermodynamic approach represented in Equation 3.3 (Table 3.2). As presented in Table 3.2, the total interfacial energy of the system was lower for Cu, with $\Delta G_{adhesion} < 0$ mJ m^{-2}. These results suggest that all studied microalgal strains are expected to adhere to Cu surfaces. On the other hand, $\Delta G_{adhesion}$ was positive for PS, SS 316, PMMA, PVC and G, in the decreasing order of free energy of adhesion. These data mean that the interaction between microalgal cells and these surfaces is not thermodynamically favoured and, consequently, it is not expected to occur. This behaviour was observed for all studied microorganisms. Regarding the thermodynamic ability for adhesion between the test materials, it is noticeable that the adhesion is thermodynamically less favourable for PS, to which $\Delta G_{adhesion}$ values ranged from 29.4±20.5 to 34.8±27.2 mJ m^{-2}.

Table 3.2. Free energy of adhesion, ($\Delta G_{adhesion}$, mJ m^{-2}) between the four microorganisms and the six studied materials when immersed in water. The values are presented as the mean±SD of two independent experiments

Surface	*C. vulgaris*	*P. subcapitata*	*S. salina*	*M. aeruginosa*
PS	34.8±27.2	29.4±20.5	30.0±10.5	32.6±25.0
SS 316	25.7±12.9	21.5±9.6	23.5±3.7	24.2±12.7
PMMA	21.8±14.5	16.6±9.3	18.7±0.7	22.8±12.5
PVC	6.9±0.1	2.7±3.6	6.15±9.7	7.3±3.5
G	5.3±12.4	1.8±9.6	3.2±5.3	4.4±14.8
Cu	-10.6±8.1	-15.4±5.6	-11.9±1.4	-16.9±7.6

A good linear relationship was obtained between free energy of hydrophobic interaction and free energy of adhesion ($R^2 \geq 0.986$), as it is possible to see from Figure 3.1. This strong correlation was expected, as the thermodynamic approach is based on the physicochemical properties of the materials and the microorganisms. Additionally, the positive relationship between free energy of hydrophobic interaction of the studied materials and free energy of adhesion corroborates the hypothesis that cell adhesion tends to occur in hydrophobic surfaces rather than on hydrophilic ones.

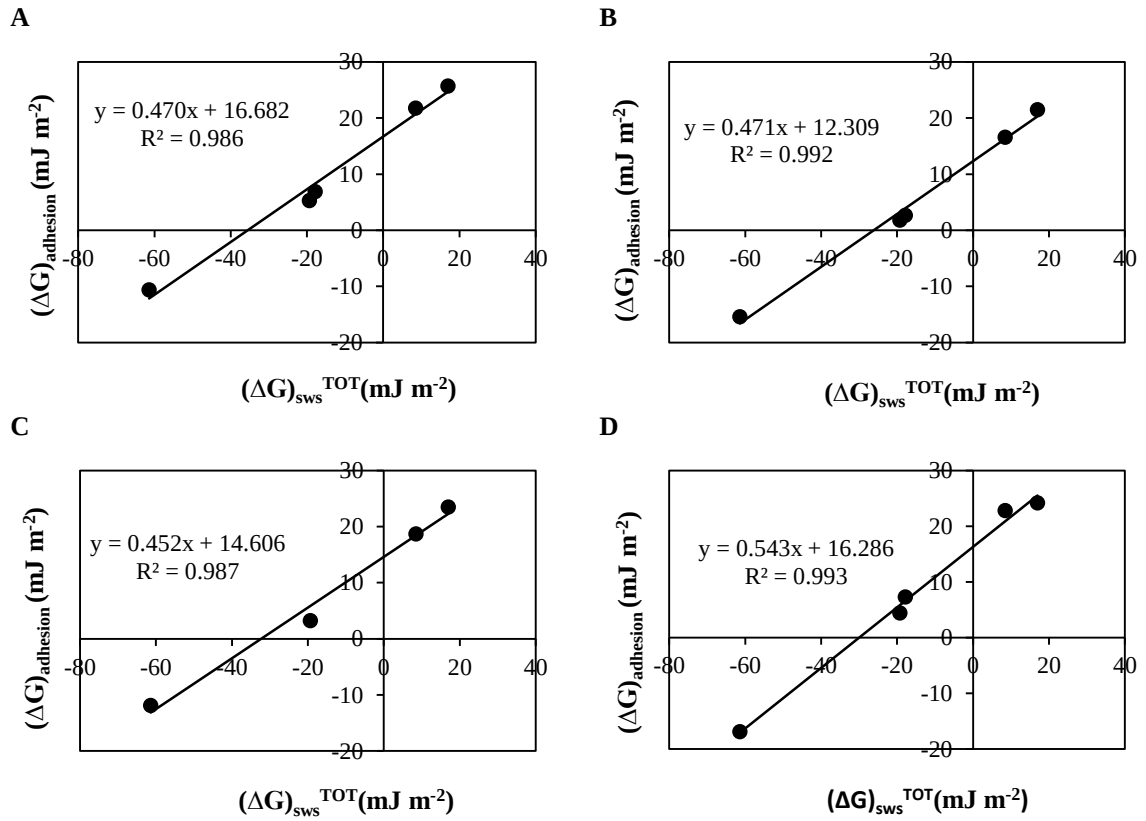

Figure 3.1. Linear regression between the free energy of adhesion ($\Delta G_{adhesion}$, mJ m^{-2}) and the free energy of hydrophobic interaction (ΔG_{sws}^{TOT}, mJ m^{-2}) of *C. vulgaris* (A), *P. subcapitata* (B), *S. salina* (C) and *M. aeruginosa* (D).

To our knowledge, this is the first study relating the free energy of adhesion between the referred microalgae and cyanobacteria and the selected materials. Therefore, it was impossible to cross data obtained in these experiments with values from other studies.

3.4. Conclusions

The free energy of hydrophobic interaction showed that SS 316 and PMMA are hydrophilic, whereas PS, PVC, G and Cu are hydrophobic surfaces. Both selected microalgae and cyanobacteria presented a hydrophilic character. Regarding the electron acceptor and donor parameters, it was possible to conclude that the selected materials and microbial surfaces are more predisposed to donate electrons than to accept them, with a predominance of the electron donor component (γ_s^-) was determined for microbial surfaces. The results of the free energy of adhesion showed a smaller value for Cu, with $\Delta G_{adhesion} < 0$ mJ m^{-2}, suggesting that microbial adhesion is more thermodynamically favoured, and it is expected to occur. On the other hand, the adhesion of the microalgal/cyanobacterial cells to the other materials is

not thermodynamically favoured. Moreover, a good correlation was obtained between the free energy of hydrophobic interaction and the free energy of adhesion ($R^2 \geq 0.986$), meaning that knowledge on surface physicochemical properties is quite important to predict the adhesion between different microorganisms and materials. However, this is only a thermodynamic prediction and a lot more factors should be taken into account, particularly the existence of extracellular appendages and the production of EPS.

CHAPTER 4

4. The effect of different surfaces and media composition on biofilm formation of selected microalgae and cyanobacteria

4.1. Introduction

Microalgal biofilms consist of a layer of cells attached to a substrate and embedded in an organic and biological matrix [51]. Formation of this complex structure, strongly depends on several factors that can be divided into biological ones, physicochemical and environmental conditions. Biological factors influencing microalgal biofilm formation include EPS production [126] and the presence of external structures able to promote microbial adhesion. Physicochemical properties of the substratum and of the cell surfaces also influence microalgal attachment. Finally, environmental conditions affecting photosynthetic biofilms include light [95], pH [101], hydrodynamic conditions [113], nutrients quality and availability [89, 104], temperature [109] and carbon source [194].

Although physicochemical properties of materials have been widely studied, as they can influence biofilm development, several authors [119, 147, 150] have reported that other factors, such as culture medium and microalgal species, can play an important role on microalgal/cyanobacterial attachment to a surface. Hultberg et al. [99] studied the effect of light quality on biofilm formation by *C. vulgaris*, showing that exposure to white, purple and blue light resulted in higher biofilm formation than that of cells exposed to red, green and yellow light. A previous study with *Chlorella* sp. showed that biofilm formation occurred only when microalgae were under shaking conditions [150]. These authors, tested different adhesion surfaces and found that polystyrene foam was the best one [150]. Some findings also suggested that, for example, the attachment of *C. vulgaris* to glass surfaces can be enhanced by the presence of bacterial films [195]. Moreover, when studying the influence of surface roughness on the adhesion of *C. vulgaris*, *Nitzschia amphibia* and

Chroococcus minutus to stainless steel and titanium, Sekar et al. [172], determined that microbial attachment to rough surfaces was higher than the one observed for smooth surfaces. In the same study, the authors concluded that *N. amphibia* adhesion was significantly influenced by pH and that the number of adhered cells was proportional to the culture density. Becker [119] also found that higher adhesion density was proportional to EPS production.

Accordingly, this chapter aims to evaluate the effect of different surfaces on biofilm formation by the microalgae *C. vulgaris* and *P. subcapitata* and the cyanobacteria *S. salina* and *M. aeruginosa*, in order to find the best surfaces for biofilm development for use in wastewater treatment. Since the composition of effluents is quite variable two different synthetic effluents were used to evaluate the effect of their composition on biofilm formation. The results obtained in Chapter 3 will be linked to those obtained in this chapter, so that the relationship between surface physicochemical properties of the surfaces and microalgal adhesion can be evaluated.

4.2. Materials and methods

4.2.1. Microorganisms and culturing conditions

The microorganisms and culturing conditions used are described in Section 3.2.1.

4.2.2. Culture media

For this experiment two different media were used: OECD test medium [178] and a synthetic medium simulating a domestic effluent (SE). Selection of these culture media was based on the following factors: (i) OECD test medium is a standard medium established by OECD for the culture of microalgae and cyanobacteria; and (ii) since this work aims the development of microalgal biofilms for application in wastewater treatment processes, a culture medium that mimics real effluents, such as SE, was required. OECD test medium is an inorganic medium presenting some macronutrients, such as inorganic nitrate and phosphate, and a wide variety of inorganic salts, the trace elements required for microalgal growth. Composition of this culture medium is the same as the one described in Section 4.2.1. On the other hand, the SE used in this study is a modified version of the one proposed by Gebara [196] and has the following composition (per litre): 300 mg $C_6H_{12}O_6$, 62 mg $NaNO_3$, 10 mg $MgSO_4 \cdot 7H_2O$,

11 mg KH_2PO_4, 1 mg $MnSO_4 \cdot H_2O$, 0.46 mg $CaCl_2$, and 0.05 mg $FeCl_3 \cdot 6H_2O$. This culture medium also presents inorganic sources of nitrogen and phosphorus. However, it presents an organic carbon source: glucose.

4.2.3. Adhesion assays

Adhesion tests were performed with *C. vulgaris*, *P. subcapitata*, *S. salina* and *M. aeruginosa.* The biofilm formation was performed as described in Meireles et al. [197] with some modifications. Coupons of PS, SS316, PMMA, G, PVC and Cu were used as adhesion surfaces. The dimensions of the coupons were approximately 1.0×0.9 cm, with a thickness ranging from 0.1 to 0.2 cm. The effect of media composition was also studied. The coupons were washed with detergent and sterile water, and sterilized under UV light during 30 min. Then, the coupons were inserted in 12-well microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium).

Microalgal suspensions were centrifuged at 16800 *g* for 15 min (5810 R Eppendorf, Germany) in two different tubes. After centrifugation, the supernatant was discarded and the respective medium was added to each tube in order to have a final concentration of about 1.0×10^6 cells mL^{-1}. Then, 3 mL of each cell suspension was added to the respective well with the coupon already inserted. The biofilm formation was allowed to occur for 24, 72 and 168 h at room temperature and constant light intensity (140.5±0.8 µE m^{-2} s^{-1}). The plates were placed in a KS 130 Basic orbital shaker (IKA Werke, Germany), as it is shown in Figure 4.1, with constant agitation of 160 rpm.

Figure 4.1. Experimental setup used for biofilm formation on coupons of different materials.

After the incubation period, the coupons were removed from the plates and placed in 1 mL or 3 mL of saline solution depending on the time of incubation. Then, the cells were removed from the coupons by vigorously vortex for 1 min. For the adhesion tests, two replicates were performed for each coupon in each condition.

4.2.4. Quantification of adhered cells

Biofilm growth was monitored through optical density (OD) measurements and determination of the number of colony forming units (CFUs) for each time point.

To assess the OD profile, 100 µL of each suspension was transferred to a 96-well microtiter plate (Orange Scientific, Braine-l'Alleud, Belgium) and the OD was measured at 750 nm in a SynergyTM HT 96-well microplate reader (Biotek Instruments, Inc., USA). For this assay, triplicates of each sample were performed. The adherence of microalgae and cyanobacteria to surfaces was then classified using the approach of Stepanović et al. [198], which can be described as follows: (i) non-adherent (0), if OD≤ODc; (ii) weakly adherent (+), if ODc<OD≤2×ODc; (iii) moderately adherent (++), if 2×ODc<OD≤4×ODc; and (iv) strongly adherent (+++), if 4×ODc<OD. The ODc is the cut-off of the OD and is defined as three standard deviation values above the mean of the OD of the negative control [198].

In order to determine the number of CFUs, the necessary dilutions were prepared and plated on modified Bold Basal medium [199] supplemented with agar (1.5%, m/v) with the following composition (per litre): 0.75 mg $NaNO_3$, 25 mg $CaCl_2 \cdot 2H_2O$, 75 mg $MgSO_4 \cdot 7H_2O$, 20 mg $FeCl_3 \cdot 6H_2O$, 20 mg $Na_2EDTA \cdot 2H_2O$, 75 mg K_2HPO_4, 75 mg K_2HPO_4, 175 mg KH_2PO_4, 20 mg NaCl, 185 mg H_3BO_3, 415 mg $MnCl_2 \cdot 4H_2O$, 3 mg $ZnCl_2$, 0.01 mg $CuCl_2 \cdot 2H_2O$, 7 mg $Na_2MoO_4 \cdot 2H_2O$ and 1.5mg $CoCl_2 \cdot 6H_2O$. This assay was performed according to the motion drop method [200], using duplicates for each suspension. The presence or absence of cells on the studied coupons was calculated as the CFU cm^{-2} and represented along the time, according to Equation 4.1:

$$\text{CFU } cm^{-2} = \frac{\frac{N}{V_P} \cdot V_S \cdot D}{A} \qquad 4.1$$

where N is the number of CFUs, V_P is the volume plated (0.01 mL), V_S is the volume of saline present in each tube (1 or 3 mL), D is the dilution and A is the total area of each coupon (cm^2).

4.2.5. Statistical analysis

The number of CFUs on each surface at each time point for the different studied media was analysed using paired-samples *t*-test from the statistical software SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Statistical calculations were based on a confidence level ≥95% ($p<0.05$ was considered statistically significant).

4.3. Results and discussion

4.3.1. Microalgal/Cyanobacterial adhesion on different surfaces

The quantification of microalgal biofilm remains a challenge, since there are many methods described in the literature, mainly for bacteria. In this study, several methods were used, such as Fluorescein DiAcetate (FDA) assay and epifluorescence microscopy (data not shown) for viability assessment, chlorophyll extraction and quantification (see Figure A.1, Annexe A.3) and OD measurements at 750 nm of the resuspended cells (see Table A.1, Annexe A.1). However, the fluorescence loss or the interference of some compounds, the small size of microalgal and cyanobacterial cells and the low cell concentrations determined at the surface of the coupons led to the selection of CFUs quantification as a reliable method.

Table 4.1 presents the number of CFUs obtained for each material tested and each culture media. It was not possible to quantify the CFUs for *P. subcapitata*, since there was no biofilm formation using this microalga and, consequently, there were no cells attached to the studied surfaces. Initial adhesion was determined after 24 h and significant differences ($p<0.05$) were observed in all materials. Although the results are not totally in accordance with each other, the degree of microbial adhesion, with few exceptions, was found to follow the sequence PVC > SS 316 > PS > PMMA> G > Cu.

Table 4.1. Logarithm of the number of colony forming units per cm^2 (log CFUs cm^{-2}) of the studied microalgae and cyanobacteria attached to PS, SS 316, PMMA, G, PVC and Cu, at time 24, 72 and 168 h, on different media. Values are means±SDs of two independent experiments

	24 h		72 h		168 h	
	SE	OECD	SE	OECD	SE	OECD
C. vulgaris						
PS	2.8±0.2	2.5±0.1	0.6±0.2	1.6±0.1	0.5±0.2	1.6±0.0
SS 316	2.6±0.2	2.8±0.1	0.5±0.1	1.4±0.0	*	*
PMMA	2.2±0.1	2.2±0.2	0.2±0.1	1.2±0.1	0.9±0.1	1.3±0.2
G	2.3±0.0	2.3±0.0	0.3±0.1	1.4±0.1	*	1.5±0.0
PVC	2.7±0.1	2.7±0.0	0.8±0.2	1.3±0.1	0.5±0.2	1.2±0.1
Cu	1.2±0.0	1.6±0.1	1.0±0.2	0.6±0.0	*	*
S. salina						
PS	2.4±0.1	2.4±0.1	2.7±0.0	2.7±0.0	2.8±0.0	2.6±0.0
SS 316	2.4±0.0	2.6±0.0	2.9±0.1	1.6±0.1	2.7±0.0	3.0±0.0
PMMA	1.0±0.2	2.6±0.0	2.7±0.1	2.3±0.1	2.4±0.0	2.6±0.1
G	1.4±0.1	2.6±0.1	2.2±0.0	2.7±0.0	2.3±0.0	2.6±0.0
PVC	0.9±0.1	2.8±0.0	2.8±0.1	2.8±0.0	2.8±0.1	2.9±0.0
Cu	*	*	2.0±0.1	2.1±0.0	*	*
M. aeruginosa						
PS	2.6±0.1	2.5±0.2	2.8±0.0	2.7±0.0	1.3±0.0	2.5±0.0
SS 316	2.3±0.1	2.4±0.1	1.5±0.1	2.7±0.0	3.0±0.0	1.8±0.0
PMMA	3.8±0.0	3.7±0.0	*	2.2±0.1	2.6±0.1	3.0±0.0
G	3.7±0.0	3.7±0.2	2.4±0.0	2.0±0.9	2.6±0.0	2.9±0.0
PVC	3.1±0.0	3.2±0.0	2.7±0.1	2.7±0.0	2.7±0.1	2.9±0.0
Cu	*	*	*	*	*	*

* Value above the detection limit.

Comparing adhesion ability within the studied microorganisms, a significant difference (p<0.05) was detected between *C. vulgaris* and *M. aeruginosa*, and also between *M. aeruginosa* and *S. salina. M. aeruginosa* presented the highest ability to adhere, followed by *S. salina*, *C. vulgaris* and *P. subcapitata*. Also, the overall results showed that adhesion was influenced most mostly by the type of material used, rather than by the culture media or microorganism.

Gonçalves et al. [164] studied surface the physicochemical properties and the zeta potential of the same microalgal/cyanobacterial species used in this work, determining zeta potential values ranging from −35.4±0.4 to −48.1±0.9 mV. Net zeta potential gives information about the functional groups present on cell surfaces and the stability of microbial suspensions. Taking into account the results reported in this study, it is possible to conclude that functional groups on the microorganisms' surface were deprotonated. Moreover, the high net zeta potential values indicate the predominance of repulsive forces over van der Waals forces and, therefore, a good stability of cells in dispersion, meaning that the formation of aggregates is

not expected to occur [164, 201]. These data explain the low values of CFUs determined in this study. In other studies reported in the literature, higher orders of magnitude were determined for this parameter [147, 172, 202].

OD measurements at 750 nm allowed the classification of adherence according to the method proposed by Stepanović et al. [198]. As it can be observed from Table A.2 (Annexe A.2), the OD was higher at 24 h for all microorganisms, meaning that biofilm formation occurred within the first 24 h, which has already been reported in the literature [89, 147, 203]. At 72 h, the OD decreases, which is probably due to the detachment and sloughing of cells from the coupons. The same behaviour was observed by Menicucci Jr [203], when studying the adhesion of *B. braunii* to an aluminium coupon. After one week (168 h), an increase in the OD values was observed, but the values were lower than those obtained after 24 h of incubation. Thus, regarding the data presented in Table 4.1, it is noticeable that the trend observed in OD values is in accordance with CFUs determined for the same time points, with exception of Cu, which will be further discussed. Figure A.2 from the Annexe A.4 also shows this behaviour. The classification proposed by Stepanović et al. [198] was then used to more easily compare adhesion ability of the selected microorganisms to the different materials. These results are shown in Table 4.2.

As it can be observed from Table 4.2, the studied microorganisms, materials and culture media have influenced microbial adherence. According to this approach, *S. salina* showed to be strongly adherent to the majority of the studied surfaces. With a few exceptions, *C. vulgaris* and *M. aeruginosa* presented a similar behaviour in terms of adherence to the studied materials. Moreover, a strong degree of adherence was assessed for all microorganism-Cu combinations. Nevertheless, comparing data from Table 4.***1*** and Table 4.***2***, it is possible to see that the Stepanović classification is not in accordance with the CFUs quantification. While the number of CFUs is a quantitative method, the Stepanović approach gives a qualitative classification about microalgal adhesion. Besides, Stepanović classification is based on OD measurements, which has proved not to be a reliable method for biofilm quantification. In fact, OD can be influenced by oxidation reactions, which happened to Cu coupons, since a blue colour was detected on their surface.

Table 4.2. Adhesion ability of the studied microalgae and cyanobacteria to PS, SS 316, PMMA, G, PVC and Cu, at 24 h, using different culture media. These results were obtained through the adherence classification proposed by Stepanović et al. [198]

	C. vulgaris		***P. subcapitata***		***S. salina***		***M. aeruginosa***	
	SE	OECD	SE	OECD	SE	OECD	SE	OECD
PS	0	+	+++	0	+	+++	0	++
SS 316	++	+++	+	+	+++	+++	+++	+++
PMMA	+	+++	0	0	+++	++	+++	+++
G	++	+++	+	+++	+++	+++	+++	+++
PVC	0	+	0	0	+++	0	0	0
Cu	+++	+++	+++	+++	+++	+++	+++	+++

4.3.2. The effect of media composition on microalgal/cyanobacterial adhesion

From Table 4.1 it is possible to observe that the number of CFUs determined when using OECD test medium was higher than the one determined when SE was used as culture medium. These results were expected, since SE presents in its composition an organic source of carbon: glucose. Microalgae and cyanobacteria are mixotrophic, meaning that they can use both light and organic carbon as energetic source. Since OECD test medium does not present such a valuable nutrient, microalgae are not in their optimal growth conditions on that medium. According to the literature, there is an enhancement of microalgal/cyanobacterial biofilm development, nutrient uptake and lipid accumulation under stress and nutrient deprivation conditions [204, 205], explaining the differences observed in microbial adhesion when using these culture media. Irving and Allen [147] also found that the attachment rates of *C. vulgaris* to glass wools were affected by culture medium composition.

However, a few exceptions were found, since in some cases, there was a higher number of CFUs in SE comparing to OECD test medium. These results can be explained by the fact that microalgae and cyanobacteria produce EPS that are the architectural network of biofilms, protecting cells from damage and promoting internal cells communication [206]. So, in those cases an increased release of EPS may have occurred, being responsible for the attachment and consequent biofilm development onto the studied surfaces. Another possibility is that the roughness of the materials, reported as a key factor influencing microalgal attachment, was not the same, leading to contradictory findings.

4.3.3. Relationship between surface physicochemical properties and microalgal/cyanobacterial adhesion

Comparison between the thermodynamic prediction (Table 3.2 from Chapter 3) and the real adhesion assays shown in Table 4.1 has confirmed that microalgal and cyanobacterial adhesion cannot be surely predicted by thermodynamic approaches. Although $\Delta G_{adhesion}$ of PS, SS 316, PMMA, G and PVC assumed a positive value for both microalgae and cyanobacteria, showing that the adhesion of these microorganisms to the materials referred above was thermodynamically unfavourable, the experimental data demonstrated their ability to adhere. On the other hand, data obtained from the physicochemical properties analysis indicated that all microorganisms should have higher ability to adhere to Cu surfaces. However, Cu was the surface with less adhered cells. Also, in most cases there was no growth onto Cu surfaces. Indeed, in the past few years Cu has been reported as a toxic material to microalgae [174-177]. Additionally, many authors [207-210] found that *M. aeruginosa* is more sensitive to Cu than *C. vulgaris*, which corroborates data obtained, since there were no cyanobacterial cells adhered to Cu surfaces. On the opposite *C. vulgaris* attached to Cu surfaces at 24h, although those values are lower comparing to the results obtained for the other tested materials.

There are many studies focusing on microalgal or materials physicochemical properties, but studies relying on characteristics of both are few. Furthermore, comparison between thermodynamic approaches and real adhesion experiments are even more uncommon and, usually, are related to bacterial adhesion. To our knowledge this is the first study reporting the relationship between microbial (*P. subcapitata, S. salina* and *M. aeruginosa*) adhesion prediction and biofilm formation. On the other hand, the theoretical and real adhesion of *C. vulgaris* to PS, PMMA, SS 316, G, and Cu have already been studied [58, 61, 62, 172, 202, 211]. In these studies, the thermodynamic approach was not always in accordance with the adhesion assays, proving that more factors should be accounted in the prediction models.

Considering data obtained for surface physicochemical properties and comparing with those obtained for microalgal/cyanobacterial adhesion, a linear relationship was found. As it can be seen in Figure 4.2, the correlation obtained was not good (R^2=0.066). However, there is a

negative relationship, since lower values of $\Delta G_{adhesion}$ correspond, in general, to a higher number of adhered cells.

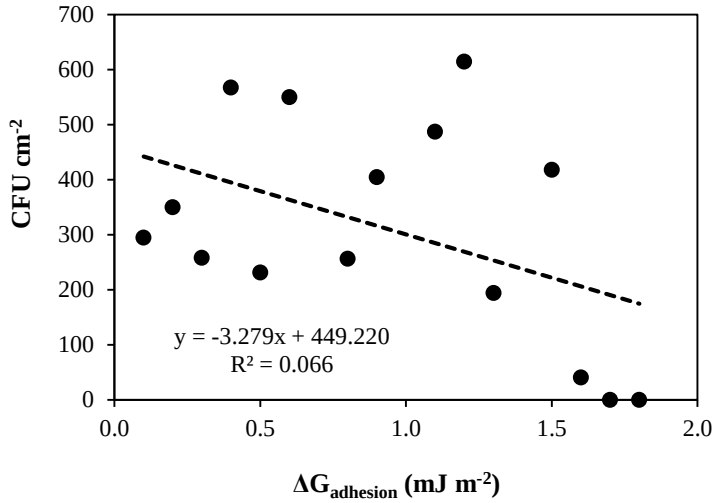

Figure 4.2. Linear regression between the number of colony forming units (CFU cm^{-2}) and the free energy of adhesion ($\Delta G_{adhesion}$, mJ m^{-2}).

The discrepancies observed between the adhesion based on thermodynamic theory and experimental results can be explained by the non-consideration of environmental and microbiological parameters in the predictive approaches. According to the DLVO theory, higher radius of particles lead to more repulsive electrostatic interactions, meaning that some biological mechanisms, such as extracellular polymers, fimbriae, prosthecae, pili or microbial flagella can benefit microbial adhesion [190, 212, 213]. Some researchers found that the attachment of microalgae/cyanobacteria may be enhanced by the presence of bacteria on substrates. Indeed, natural biofilms are mainly complex consortia of autotrophs, heterotrophs and EPS [61, 206, 214]. Surface roughness and texture have also been reported in the literature as key factors for biofilm development [172, 206, 214].

4.4. Conclusions

Although surface physicochemical properties can influence microalgal and cyanobacterial biofilm formation, there are many other factors equally important, such as the culture media and the type of surface used. In this study, biofilm formation occurred within the first 24 h, followed by detachment of cells from the surfaces (72 h) and new biofilm development (168 h). Concerning the ability of microorganisms to adhere to the studied materials,

M. aeruginosa adhered at higher extent to surfaces, followed by *S. salina* and *C. vulgaris*. *P. subcapitata* was the exception, as the results showed a lack of adhesion ability. The degree of microbial adhesion was found to follow the order PVC > SS 316 > PS > PMMA > G > Cu. A significant difference between the number of cells attached on SE and OECD test medium was determined, proving that biofilm formation can be enhanced by exposing microorganisms to nutrients stress conditions. Also, comparison between the thermodynamic theory and the experimental assays showed that adhesion is underestimated when predicted by the thermodynamic approach, and its prediction depends on many other factors apart from surface physiochemical properties.

CHAPTER 5

5. Nutrients removal using microalgal biofilm reactors

5.1. Introduction

In the past few years, the input of nutrients in water courses has increased, due to industrialization, agricultural practices and alterations in humans daily life routines [2]. These activities have a large impact on the ecosystem, leading to the pollution and eutrophication of hydric resources and the degradation of the ecosystem. Accordingly, many studies have focused on nutrients removal from wastewater, mainly phosphorus and nitrogen, by means of chemical, biological or physical treatments [3, 161]. However, the existing methods are expensive, time-consuming or even inefficient, whereby alternative methods are required.

For more than half a century, researchers have investigated the concept of growing microalgae on wastewater [215-218]. Although the usage of microalgae to remove nutrients from wastewater streams can be a great choice, the harvesting of microalgae remains a critical point [6]. In this sense, immobilization methods have been applied to microalgae to solve the problems associated to suspended growth systems [2, 68]. Accordingly, microalgal biofilms emerged as a cost-effective and ecologically safe solution, as they can remove nutrients and heavy metals from wastewaters and also produce biomass that can be used for the production of valuable compounds.

There are many reactors using microalgal biofilms for wastewater treatment. The rotating algal biofilm reactor (RABR) consists in a cylinder connected to a motor through a shaft. The cells attach to a substratum clothed around the cylinder, growing on its surface while the cylinder rotates and contacts partially with water [138, 219]. This device allows a simple and cheap harvesting of microalgae, by just scrapping off the surface. Moreover, the RABR

presents other advantages, such as high nutrients removal rates, good gas exchange and possible advantages from the intermittent light:dark cycles [7, 219]. Johnson and Wen [150] developed a polystyrene rocker system, using different materials for microalgal and cyanobacterial attachment. Briefly, a coupon of the selected material is placed into a growth chamber, incubated with algal cell suspension. Then, the chamber is placed on a rocking shaker and the supporting material is alternately submerged on wastewater, with constant light intensity. After the mat formation, biomass is harvested by scrapping of the surface. Although their objective was to enhance biofuel production, this mechanism allowed nutrients removal efficiencies of about 70 to 100%. Algal Turf Scrubber (ATS) is one of the most used reactors for wastewater treatment. This technology was first engineered by Adey and Hackney [220] and has been used ever since in several studies by other authors. The ATS consists in a polystyrene mesh, on laboratory scale, or a nylon netting, on pilot or field scale [51, 221]. Algae attach to this platform and grow, with constant light and wave surge, preventing the creation of a boundary layer that limits light penetration and nutrients exchange [151]. Several studies showed good nutrients removal (36-93%) and high biomass productivities (5 to 20 g m^{-2} d^{-1}) using this system [151, 222, 223]. Another design, the polycarbonate flow lane, is a laboratory photobioreactor, especially engineered for the cultivation of phototrophic biofilms. It allows the assessment of nutrients removal and, simultaneously, the control of external parameters, such as photosynthetic photon flux density, temperature and flow velocity. With high biomass productivities, maximal phosphorous removal rates can be obtained [149].

This chapter aims to compare two different biofilm production systems, a stirred tank reactor (STR) and a rotating disk reactor (RDR), for nutrients (mainly nitrogen and phosphorus) removal from culture medium. The microalga *C. vulgaris* was selected as it is a well-established microorganism, one of the most studied microalgae, and mostly used at scientific research and industrial levels. Also, *C. vulgaris* has demonstrated high ability to remove nitrogen, phosphorus, and chemical oxygen demand. This microorganism is widely used, since it can grow rapidly and under hard conditions [224, 225].

5.2. Materials and methods

5.2.1. Microorganisms and culturing conditions

The microalga *C. vulgaris* and the culturing conditions used are described in Section 3.2.1.

5.2.2. Stirred tank reactor setup and sampling

In this experiment, the biofilm was developed in 6-well microtiter plates (Orange Scientific, Braine-l'Alleud, Belgium). Coupons of SS 316 (dimensions of 2×2.1×0.1 cm) were selected as adhesion surfaces, due to the high ability of microalgae to adhere to this material, as verified in Chapter 4. A microalgal suspension with a final concentration of 1.0×10^6 cells mL^{-1} was added to each well of the microtiter plates where the coupons, sterilized through UV for 30 min, were already placed. The biofilm was allowed to develop for 72 h at 80 rpm. Then, 24 coupons with biofilm were carefully placed on the clips of the reactor using a sterile tweezer. The reactor used was a STR made of PMMA with a working volume of 4 L and magnetically agitated, similar to the one used by Ferreira et al. [226] (Figure 5.1). The reactor was then filled with OECD test medium. This experiment was carried out in batch mode for 18 days, with constant light intensity of 142.9±10.0 µE m^{-2} s^{-1}.

After 72 h of incubation, a coupon with biofilm was analysed by scanning electron microscopy (SEM). Before SEM observations, the sample was fixed, successively dehydrated and dried, according to the method described by Gomes et al. [227] method. A coupon without biofilm was also analysed in order to see the differences after biofilm formation. The reactor was subjected to constant light irradiance (196.6±10.4 µE m^{-2} s^{-1}). The pH was measured at the beginning and the end of the experiment using a HI 8424 pH meter (HANNA Instruments, USA).

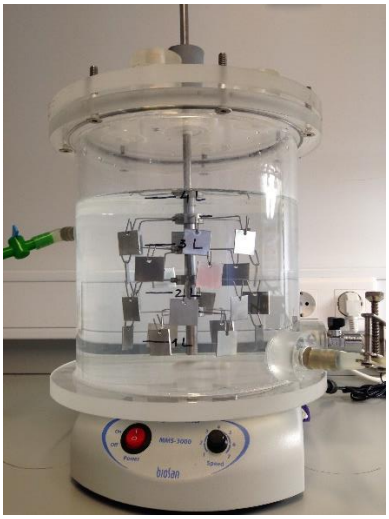

Figure 5.1. Experimental setup for cultivation of microalgal biofilm on a stirred tank reactor (STR).

5.2.3. Rotating disk reactor setup and sampling

For this assay, a RDR was used. The RDR is a 5-L (PMMA) reactor with four separate sections and two rotating disks in each one, rotated through a shaft connected to a cylinder. For the biofilm development, *C. vulgaris* was used for the reasons stated above. In order to test the effect of hydrodynamic conditions on nutrients removal, two experiments were performed at two different stirring speeds (2.3 and 6.9 rpm). The experimental setup of the RDR is presented in Figure 5.2. The reactor was operated in batch with OECD test medium for 13 days (for each experiment).

At each time point, a sample was collected from the reactor and the OD was measured at 750 nm using a V-1200 spectrophotometer (VWR, Germany). The pH of the suspension was determined daily using a HI 8424 pH meter (HANNA Instruments, USA). The dissolved oxygen concentration (mg L^{-1}), along with the temperature inside the reactor were also daily monitored, using a 340i Handheld meter (WTW, Germany). The reactor was subjected to constant light irradiance (57.9.±2.9 µE m^{-2} s^{-1}).

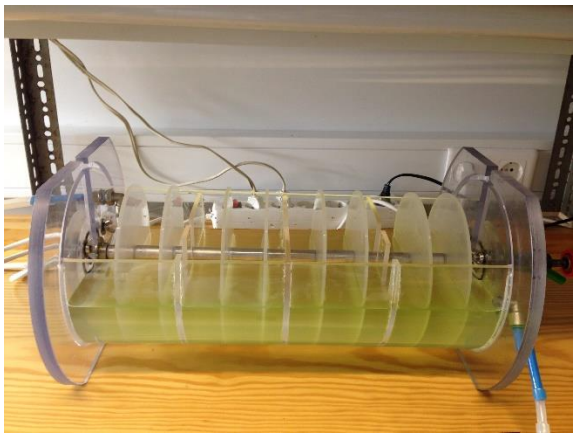

Figure 5.2. Experimental setup for cultivation of microalgal biofilm on a rotating disk reactor (RDR).

5.2.4. Nutrients removal

Nutrient removal was determined by quantification of nitrogen and phosphorus in the culture medium of both reactors. For each analytical assay, one-millilitre samples from each reactor were collected for all time points of the experiment. Samples were centrifuged at 16500 *g* for 10 min and supernatants were stored at -20 °C until being analysed.

Nitrate concentration was determined according to the method described in the literature [168, 228]. At first, the supernatants were thawed, diluted approximately 20 times in distilled water and filtered using 0.22-µm cellulose acetate membranes (Orange Scientific, Belgium). After filtration, absorbance of the samples was measured at 220 nm using a quartz cuvette in a T80 UV/VIS spectrophotometer (PG Instruments, UK). Distilled water previously filtered was used as blank. The calibration curve was determined by preparing $NaNO_3$ standards with concentrations ranging from 5 to 500 mg L^{-1} and submitting them to the same procedure as the analysed samples. Samples were analysed in triplicates. The relationship between absorbance at 220 nm, *y*, and the nitrate concentration, *x*, was established by linear regression (see Figure A.3, Annexe A.5).

The inorganic phosphate quantification was performed by measuring absorbance at 820 nm of a phosphomolybdate complex formed by reaction of inorganic phosphate with ammonium molybdate, as proposed by Lee et al. [229]. In this method, 60 µL of each of the thawed supernatants was pipetted into a well of a 96-well microtiter plate (Orange Scientific, USA) and then 140 µL of the reaction mix was added. The reaction mix was prepared by adding 1 part of reagent 1 (10% ascorbic acid) to 6 parts of reagent 2 (0.42% $(NH_4)_2MoO_4 \cdot 4H_2O$ in

1 N H_2SO_4). The microtiter plate was then incubated at 37 °C for 1 h and the absorbance was measured at 820 nm in a SynergyTM HT 96-well microplate reader (Biotek Instruments, Inc., USA). The blank was measured by repeating the procedure using distilled water. To determine the calibration curve, standards of KH_2PO_4 with concentrations ranging from 1 to 60 mg L^{-1} were submitted to the same procedure. Samples were analysed in triplicates. The relationship between absorbance at 820 nm, *y*, and the phosphate concentration, *x*, was established by linear regression (see Figure A.4, Annexe A.6).

The nitrogen and phosphorus removal rates were determined using the following equation:

$$\%\mathrm{R} = \frac{\mathrm{C}(\mathrm{t}_0) - \mathrm{C}(\mathrm{t})}{\mathrm{C}(\mathrm{t}_0)} \times 100 \quad (5.1)$$

where $C(t_0)$ is the nutrient concentration (phosphorus/nitrogen) in mg L^{-1} of the sample determined in the beginning of the experiment and $C(t)$ is the nutrient concentration of the sample (phosphorus/nitrogen) in mg L^{-1} determined at time t.

Nutrients uptake rates were then determined by fitting the experimental data, corresponding to the time-course evolution of nutrients concentration, to the modified Gompertz model [230]. This model has already been used to describe microalgal [231-234] and bacterial [230] growth and was here simplified to determine nutrients removal kinetics, as represented in Equation 5.2:

$$\mathrm{C}(\mathrm{t}) = \mathrm{C}(t_0) + \left(\mathrm{C}(\mathrm{t_f}) - \mathrm{C}(\mathrm{t_0})\right) \times \exp(-\exp[\mathrm{k} \times (\lambda - \mathrm{t}) + 1]) \quad (5.2)$$

where $C(t)$ is the time-course evolution of nutrient (N or P) concentration, k is the nutrients uptake rate (mg L^{-1} d^{-1}) and λ is the lag time (d). The kinetic parameters, k and λ, were determined by minimizing the sum of squared residuals using the Solver supplement of Microsoft Excel 2013. The quality of the model fit was evaluated through analysis of the coefficient of determination (R^2).

5.2.5. Statistical analysis

The nutrients concentration at each time point for each reactor were analysed using paired-samples *t*-test from the statistical software SPSS 22.0 (SPSS Inc., Chicago, IL, USA).

Statistical calculations were based on a confidence level ≥95% (p<0.05 was considered statistically significant).

5.3. Results and discussion

5.3.1. Biofilm growth

The morphological changes on the surface of SS 316 coupon after the biofilm formation were analysed by SEM (Figure 5.3). The coupon of SS 316 used as control appears to be a rough surface, since there are many signs of wrinkles on the topography of the surface.

Regarding the images after biofilm formation, it is noticeable that cells are uniformly spread throughout the coupon, forming a mantle. Moreover, biofilm is formed by round shaped cells with a uniform diameter: 4.1±0.8 µm. The micrograph with higher magnification (F) shows the presence of some EPS. It has been reported in the literature [125, 206, 235] that EPS

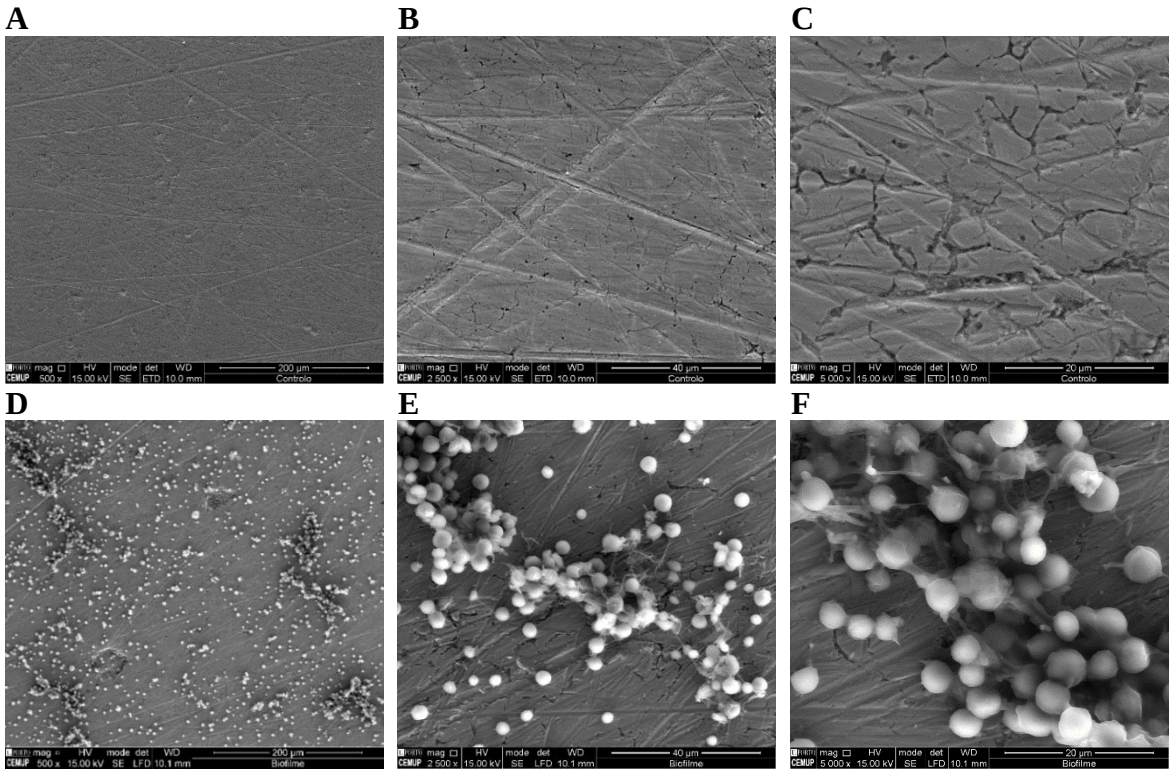

Figure 5.3. SEM micrographs of a control (A, B and C) and the microalgal biofilm attached on a SS 316 coupon (D, E and F). Micrographs A and D have a magnification of 500× (scale bar=200 µm), B and E have a magnification of ×2500 (scale bar=40 µm) and C and F have a magnification of ×5000 (scale bar=20 µm).

sticky nature helps in microbial adhesion to surfaces, as it forms a polymer network that interconnects and immobilizes biofilm cells. Furthermore, some findings suggest that the morphology and architecture of biofilm is regulated mainly by EPS [236].

The SEM analysis in conjunction with Energy Dispersive Spectroscopy (EDS) allowed the assessment of the chemical composition of the formed biofilm. The EDS spectrum analysis (see Figure A.5, Annexe A.7) showed that biofilm is mainly composed by carbon, with a few percentages of oxygen and nitrogen, the typical composition of a biofilm.

5.3.2. Nutrients removal profile using the STR

The nitrogen and phosphorus removal profile obtained for the STR is presented in Figure 5.4. During the first 9 days, nitrogen concentration values remained almost the same. After 10 days of the beginning of the experiment, biofilm starts to remove nitrogen, reaching an overall removal efficiency of 34.5%. Regarding Figure 5.4.B, it is possible to observe that although phosphorus removal started a little bit earlier (day 8), the removal efficiency achieved at the end of the experiment was similar (35.2%).

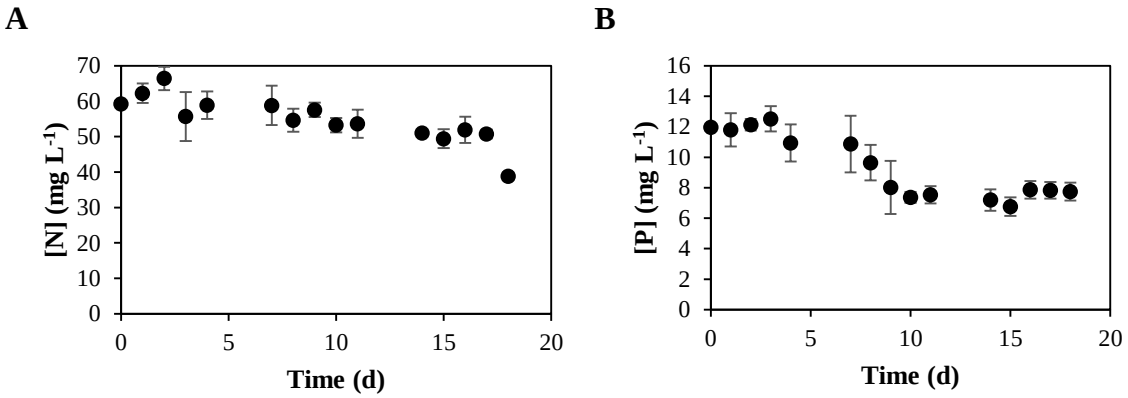

Figure 5.4. Nitrogen (A) and phosphorus (B) concentrations in OECD test medium when culturing *C. vulgaris* in the STR. Values are presented as the mean±SD of two independent experiments.

Also, the pH raised from 7.0 in the beginning to 10.6 at the end of the experiment. This change in pH was expected and will be further discussed.

Moreover, the poor EPS production observed in Figure 5.3, as well as the homogeneous distribution of microalgal cells in the coupons, can also explain the detachment of microalgal cells from the coupons observed after a few days of the beginning of the experiment. Since the matrix that helps cells in the adhesion process is small compared to the overall biofilm

formed, when exposed to agitation, cells could easily detach from the material and grow in suspension. So, the low nutrients removal observed in Figure 5.4 is due to microalgal suspension instead of microalgal biofilm and the increase in pH observed can be attributed to microalgal growth in suspension.

To our knowledge, this is the first study reporting nutrients removal by using coupons with biofilm, whereby the results obtained in this work cannot be compared to data from the literature. However, the nutrients removal efficiencies were very low and it is known that it would not be feasible for application in real wastewater treatment plants.

5.3.3. Nutrients removal profile using a RDR

In order to determine the kinetics of nutrients removal by a RDR using microalgal biofilms, two different conditions were tested: (i) a lower stirring speed of 2.3 rpm (experiment 1); and (ii) a higher stirring speed of 6.9 rpm (experiment 2). The pH and dissolved oxygen concentration were analysed along time and their profile are presented in Figure 5.5 for the two experiments performed.

As it can be observed in Figure 5.5, the dissolved oxygen concentration increased from 5.4 to 8.6 mg L^{-1} and from 5.7 to 8.0 mg L^{-1} in experiments 1 and 2, respectively. Concerning the pH profile, there was a raising, ranging from 6.7 to 9.3 and from 7.1 to 9.7 in experiments 1 and 2, respectively. According to the literature [172], microalgae are at optimal growth conditions in the pH range between 6 and 9, which is in accordance with the data obtained. These results were expected since during microalgal growth they consume CO_2 and produce oxygen through photosynthesis. Also, the rapid consumption of CO_2 during photosynthesis cause pH gradients [237, 238], since as CO_2 decreases, the equilibrium moves towards to counteract CO_2 losses, leading to the formation of OH^- ions, thus raising the pH [102, 239].

The OD of the planktonic was also monitored during experiments (see Figure A.*6*, Annexe A.8). Analysing the data obtained, it was possible to observe an overall decreasing trend of OD. This fact was expected, microalgal cells have deposited, forming a tight biofilm on the walls and at the bottom of the reactor (see Figure A.*7*, Annexe A.9). Additionally, in both experiments, biofilm formation in the disks can be considered negligible, meaning that

probably, the stirring speed was not enough to promote microalgal attachment to the reactor disks.

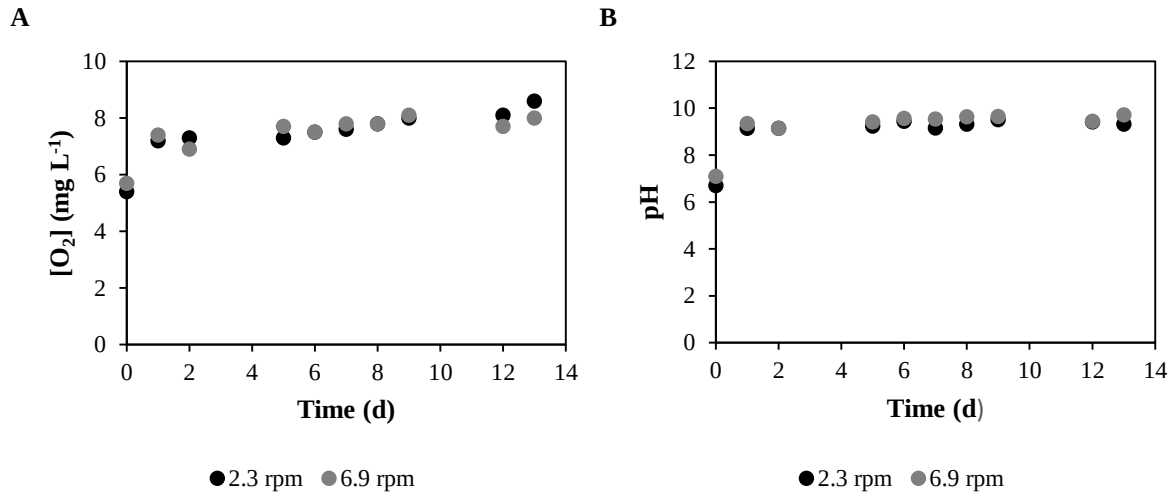

Figure 5.5. Dissolved oxygen concentration (A) and pH (B) profiles obtained when culturing *C. vulgaris* in the RDR under different stirring speeds (2.3 and 6.9 rpm).

Concerning nutrients uptake by *C. vulgaris*, Figure 5.6 shows that the nitrogen concentration in experiment 1 (2.3 rpm) decreased from 54.4 to 16.6 mg L^{-1}, while phosphorus concentration went from 12.6 to 1.1 mg L^{-1}. On the other hand, in experiment 2 (6.9 rpm) there was a decrease in nitrogen and phosphorus concentrations from 51.4 to 9.9 mg L^{-1} and 13 to 0.4 mg L^{-1}, respectively.

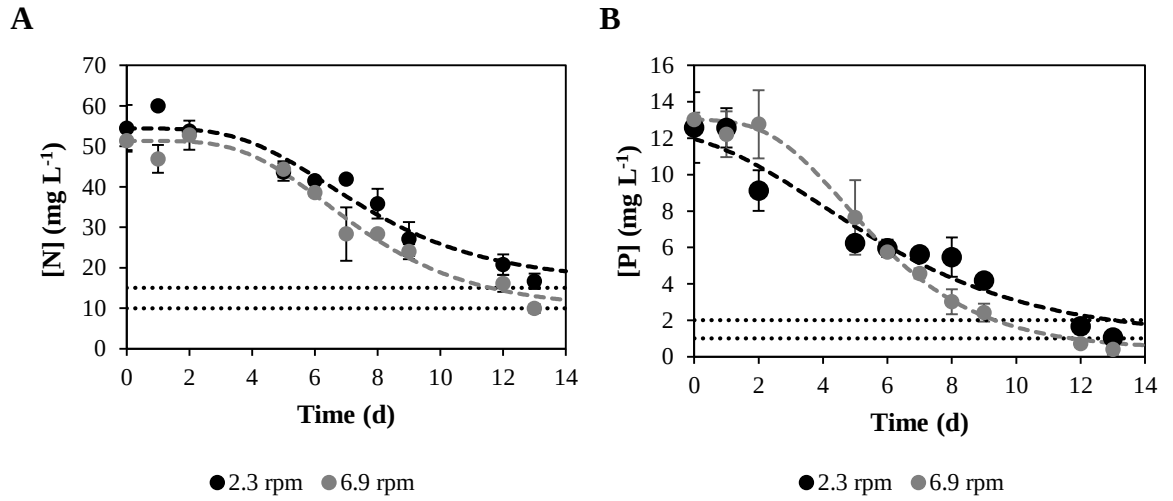

Figure 5.6. Nitrogen (A) and phosphorus (B) concentrations in OECD test medium when culturing *C. vulgaris* in the RDR under different stirring speeds (2.3 and 6.9 rpm). The horizontal dotted lines indicate the target values for the effluent according to the EU legislation. The dashed curved lines correspond to the model fit of the modified Gompertz model. Values are presented as the mean±SD of two independent experiments.

The Gompertz model was applied to both experiments to determine the removal kinetic parameters. Data obtained from this analysis is presented in Table 5.1.

Table 5.1. Kinetic parameters of the modified Gompertz model and nutrients removal efficiencies for the experiments performed in the RDR

	Experiment 1 (2.3 rpm)		**Experiment 2 (6.9 rpm)**	
	N	**P**	**N**	**P**
k (mg L^{-1} d^{-1})	0.35	0.27	0.38	0.43
λ (d)	3.59	0.25	3.71	2.34
%R	69.4	91.6	80.7	96.9
R^2	0.955	0.946	0.972	0.996

The validation of the method was assessed through linear regression coefficient (R^2) between the predicted values from the modified model and the experimental results. The coefficient of determination presented values above 0.946 and, in some cases, close to 1, meaning that the modified Gompertz model fits well experimental data and it could be used to describe the nutrients removal by *C. vulgaris* biofilm from OECD test medium using a RDR. Regarding the kinetic parameters obtained from the model, it is noticeable that the nutrients uptake rate, k, was similar between experiments, with exception of phosphorus uptake in experiment 1, which presented the lowest value (0.27 mg L^{-1} d^{-1}). Concerning the lag time parameters, λ, it is possible to verify that lower values were obtained for P removal, meaning that it takes more time for microalgal biofilm to start N removal, comparing to P removal. Moreover, comparing both experiments, it can be ascertained that although a higher stirring speed conducted to higher nutrients removal efficiencies, the microalgal biofilm required more time until the beginning of N and P removal from the culture medium, since experiment 2 presented higher lag time values. Regarding N and P removal efficiencies, it can be concluded that P uptake was higher than N uptake, reaching $\%R$ above 92%. Nitrogen removal efficiencies determined for experiments 1 and 2 were 69 and 81%, respectively.

Furthermore, comparison between both experiments from RDR indicates that experiment 2 had the highest $\%R$ values. Although those differences were not statistically significant (p>0.05), experiment 2 was the only reaching EU legislation parameters (except in the case of phosphorus removal, where the limits imposed by legislation were achieved in both experiments). In fact, according to the EU legislation [240, 241], there are limits for nutrient concentrations in discharged effluents and minimum percentage load reductions. The limits for effluent discharge are: (i) a minimum percentage of reduction of 70-80% for total

nitrogen; and (ii) a minimum percentage of reduction of 80% for total phosphorus [168]. These findings suggest that the system employed in experiment 2 could be effectively used for nutrients removal in wastewater treatment plants. Additionally, these results indicate that higher stirring speeds favour nutrients removal, which was already expected since increased stirring speeds promote a better contact between nutrients and the attached cells. Nevertheless, nutrients removal efficiencies determined in this study were lower than those reported in previous studies. For example, Sukačová et al. [242], obtained phosphorus removal efficiencies of 97% after culturing a microalgae and cyanobacteria consortia for 24 h in an artificial effluent. However, there are many parameters influencing microalgal nutrients uptake, such as light intensity, photoperiod (light:dark cycles), type of wastewater, N and P loads, type of reactor and their operation mode, species composition, pH, nutrients sources, hydrodynamic conditions and temperature [89, 122].

Comparing nutrients removal profiles obtained with both studied reactors (STR and RDR), it is clear that RDR had a much better performance ($p<0.05$), removing at least 50% more nutrients than the other system. The low removal efficiencies observed in STR can be explained by low surface area comparing to the volume of the reactor. So, it would be interesting to use larger coupons in order to have more biofilm, or to use a reactor with lower capacity.

5.4. Conclusions

The RDR demonstrated to have a better performance in nutrients removal from the synthetic effluent (OECD test medium), when comparing to STR. The biofilm formed on SS 316 coupon surface presented similar cells, round shaped and with a uniform diameter (4.1±0.8 µm). Furthermore, the two experiments performed with the RDR showed that: (i) dissolved oxygen concentration and pH profiles increased due to photosynthetic biofilm growth; (ii) a higher stirring speed allowed higher N and P removal efficiencies (81 and 97%, respectively); (iii) P uptake was higher than N uptake; (iv) the modified Gompertz model fitted well the experimental data ($R^2>0.946$); and (v) in experiment 2, microalgal biofilm showed reduction percentages higher than the values established by EU legislation. Concluding, this study shows the feasibility of removing N and P from a synthetic effluent using a microalgal biofilm system.

CHAPTER 6

6. Concluding remarks and research needs

6.1. General conclusions

With increasing eutrophication of water bodies, there is an urgent need for a cost-effective and eco-friendly alternative to remove nutrients from effluents before their discharge in natural water courses. Lately, microalgal biofilms have been used due to their ability to effectively remove nutrients and heavy metals from wastewaters and also to produce biomass that can be used for other applications. Therefore, the main objectives of this work were (i) to understand the influence of surface physicochemical properties on microalgal/cyanobacterial adhesion; and (ii) to optimize nutrients removal from effluents using photoautotrophic biofilms.

In what concerns the surface physicochemical properties, the results showed that SS 316 and PMMA are hydrophilic, whereas PS, PVC, G and Cu are hydrophobic surfaces. Conversely, microalgae and cyanobacteria demonstrated to be hydrophilic. A good correlation between free energy of hydrophobic interaction and free energy of adhesion was found (R^2>0.986), meaning that surface physicochemical properties are a key factor that influences microbial adhesion to surfaces.

The biofilm formation between the selected microorganisms and surfaces occurred within the first 24 h. At time 72 h, cells detached from the coupons and at 168 h there was a re-colonization of coupons by microalgae/cyanobacteria and, therefore, a new biofilm formation. The microalga *P. subcapitata* was the only microorganism showing lack of ability to adhere. Also, the degree of microbial adhesion was found to follow the order PVC > SS 316 > PS > PMMA> G > Cu. Cu was the only surface with no cells adhered, proving once more their toxicity to microalgal/cyanobacterial cells. Comparison between the two synthetic effluents used has shown that there was higher biofilm formation when cells were exposed

to stress conditions, since OECD test medium presented more CFUs cm^{-2}. Although adhesion predictions based on surface physicochemical properties were not correct, a negative correlation was found between the thermodynamic approach and the adhesion assays, meaning that there may be many other factors that can influence biofilm formation besides surface physicochemical properties.

Regarding nutrients removal, the RDR had a better performance compared to STR. During RDR experiments, it was verified an increase in pH and dissolved oxygen concentration, due to photosynthesis resulting from biofilm growth. The modified Gompertz model was applied to model the behaviour of nutrients (N and P) concentrations in RDR experiments and to determine nutrients removal kinetics. The nutrients uptake rate ranged from 0.27 to 0.43 mg L^{-1} d^{-1} and the lag time parameter ranged from 0.25 and 3.59 d. Also, a R^2>0.946 was obtained, which means that the applied model fitted well to the experimental data. Since high removal efficiencies were obtained (80.7% for N and 96.9% for P), EU legislation limits were reached, proving that microalgal biofilm systems can be used as a cost-effective alternative for wastewater treatment.

6.2. Research needs

This work lacks a lot of other experiments that were not possible to perform due to time constraints. Regarding the experiments using different biofilm reactors, it would be interesting to repeat the experiments but extend them until the nutrients removal stabilizes and reaches a baseline in order to quantify the maximum removal efficiency that can be achieved. Although two different stirring speeds were used in this study, other stirring speeds should be evaluated, in order to understand the role of hydrodynamic conditions on biofilm formation and on the bioreactor performance. Also, more operational conditions should be tested, such as light:dark cycles, pH ranges, light intensity, temperature and nutrients loads. Moreover, since microorganisms have shown higher ability to form biofilm under stress conditions, it would be important to study the effect of nutrients deprivation on biofilm development and nutrients removal efficiency. The influence of a real effluent and its composition on nutrients removal should also be assessed, through experimental assays and SEM observations.

Apart from the operational conditions, it can be evaluated the EPS production during biofilm formation and nutrients uptake in order to notice if there is a relationship between EPS production and microbial adhesion.

Reminding the problems found with STR, new experiments with larger coupons or, otherwise, a small reactor should be performed.

Since roughness has been reported as a factor influencing microbial adhesion, it can be also investigated the biofilm development under surfaces with different grit sizes and, therefore, the nutrients removal efficiencies obtained with those biofilms.

Furthermore, the usage of mixed cultures of different microorganisms is also promising. It has been reported by a few authors that the co-culture of microalgal and cyanobacterial species can enhance biofilm growth and nutrients removal from wastewaters.

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Annexes

A.1. The OD (750 nm) profile during biofilm development on different surfaces

The OD (750 nm) measured during biofilm formation of the studied microalgae and cyanobacteria on different surfaces and different media, at 24, 72 and 168 h is presented in Table A.1.

Table A.1. OD profile of the studied microalgae and cyanobacteria attached to PS, SS 316, PMMA, G, PVC and Cu, at time 24, 72 and 168 h, on different media. Values are presented as the mean±SD of two independent experiments

	24 h		72 h		168 h	
	SE	**OECD**	**SE**	**OECD**	**SE**	**OECD**
C. vulgaris						
PS	0.000±0.000	0.004±0.007	0.001±0.001	0.010±0.009	0.044±0.005	0.063±0.019
SS 316	0.047±0.023	0.020±0.016	0.003±0.003	0.013±0.006	0.060±0.028	0.130±0.052
PMMA	0.028±0.029	0.023±0.026	0.003±0.002	0.012±0.006	0.038±0.004	0.082±0.013
G	0.057±0.016	0.017±0.014	0.028±0.020	0.050±0.036	0.175±0.056	0.205±0.041
PVC	0.006±0.006	0.004±0.003	0.001±0.001	0.003±0.002	0.041±0.003	0.059±0.005
Cu	0.211±0.070	0.383±0.296	0.161±0.025	0.105±0.600	0.152±0.015	0.238±0.127
P. subcapitata						
PS	0.036±0.050	0.002±0.004	0.007±0.004	0.070±0.072	0.002±0.003	0.013±0.013
SS 316	0.001±0.000	0.004±0.003	0.040±0.040	0.083±0.093	0.003±0.004	0.024±0.028
PMMA	0.005±0.003	0.006±0.003	0.015±0.007	0.011±0.006	0.006±0.008	0.009±0.008
G	0.016±0.020	0.106±0.094	0.025±0.036	0.084±0.076	0.008±0.011	0.124±0.139
PVC	0.007±0.005	0.005±0.003	0.008±0.004	0.027±0.022	0.010±0.014	0.012±0.009
Cu	0.342±0.80	0.170±0.042	0.512±0.201	0.265±0.069	0.074±0.038	0.089±0.089
S. salina						
PS	0.004±0.005	0.020±0.014	0.016±0.017	0.015±0.007	0.004±0.003	0.004±0.003
SS 316	0.055±0.052	0.046±0.034	0.036±0.033	0.091±0.054	0.007±0.004	0.007±0.004
PMMA	0.038±0.042	0.008±0.008	0.018±0.014	0.022±0.017	0.033±0.007	0.033±0.007
G	0.018±0.012	0.089±0.026	0.121±0.065	0.037±0.027	0.057±0.045	0.057±0.045
PVC	0.017±0.009	0.001±0.001	0.050±0.043	0.060±0.055	0.020±0.009	0.020±0.009
Cu	0.346±0.106	0.408±0.285	0.133±0.027	0.135±0.053	0.047±0.031	0.047±0.031
M. aeruginosa						
PS	0.002±0.003	0.006±0.008	0.029±0.024	0.008±0.007	0.011±0.006	0.008±0.009
SS 316	0.074±0.014	0.059±0.044	0.021±0.025	0.027±0.019	0.030±0.025	0.210±0.135
PMMA	0.016±0.013	0.035±0.020	0.010±0.013	0.004±0.005	0.004±0.002	0.059±0.041
G	0.039±0.027	0.132±0.024	0.170±0.109	0.097±0.049	0.017±0.020	0.081±0.053
PVC	0.002±0.002	0.004±0.006	0.024±0.029	0.011±0.008	0.006±0.005	0.008±0.008
Cu	0.102±0.068	0.257±0.199	0.050±0.040	0.149±0.051	0.055±0.023	0.069±0.066

A.2. Adhesion ability of the studied microorganisms

The classification proposed by Stepanović et al. [198], used to more easily compare cell's adhesion between the studied microorganisms and materials, is represented in Table A.2 for 72 h and 168 h of the experiments.

Table A.2. Adhesion ability of the studied microalgae and cyanobacteria to PS, SS 316, PMMA, G, PVC and Cu at time 72 and 168 h, using two different media, according to the adherence classification proposed by Stepanović et al. [198]

	72 h		**168 h**	
	SE	**OECD**	**SE**	**OECD**
C. vulgaris				
PS	0	0	0	0
SS 316	0	0	0	0
PMMA	0	0	0	0
G	0	0	0	0
PVC	0	0	0	0
Cu	0	0	0	0
P. subcapitata				
PS	0	+++	0	+
SS 316	+++	+++	0	++
PMMA	+	+++	0	+
G	++	+++	+	+++
PVC	0	+++	+	0
Cu	+++	+++	+++	+++
S. salina				
PS	+++	+++	0	0
SS 316	+++	+++	+	+
PMMA	+++	+++	+++	+++
G	+++	+++	+++	+++
PVC	+++	+++	+++	+++
Cu	+++	+++	+++	+++
M. aeruginosa				
PS	+++	+++	0	+
SS 316	++	+++	++	+++
PMMA	+	++	0	+++
G	++	+++	+	+++
PVC	++	+++	0	+
Cu	+++	+++	+++	+++

A.3. Chlorophyll (a and b) quantification

The chlorophyll a and b extraction and quantification was performed according to Porra et al. [243]. The chlorophyll content of microalgal and cyanobacterial cells was quantified for the studied surfaces after 168 h of the beginning of the experiment (Figure A.*1*). Moreover, it was not possible to quantify chlorophyll content of *P. subcapitata*, since there was no biofilm formation by this microalga.

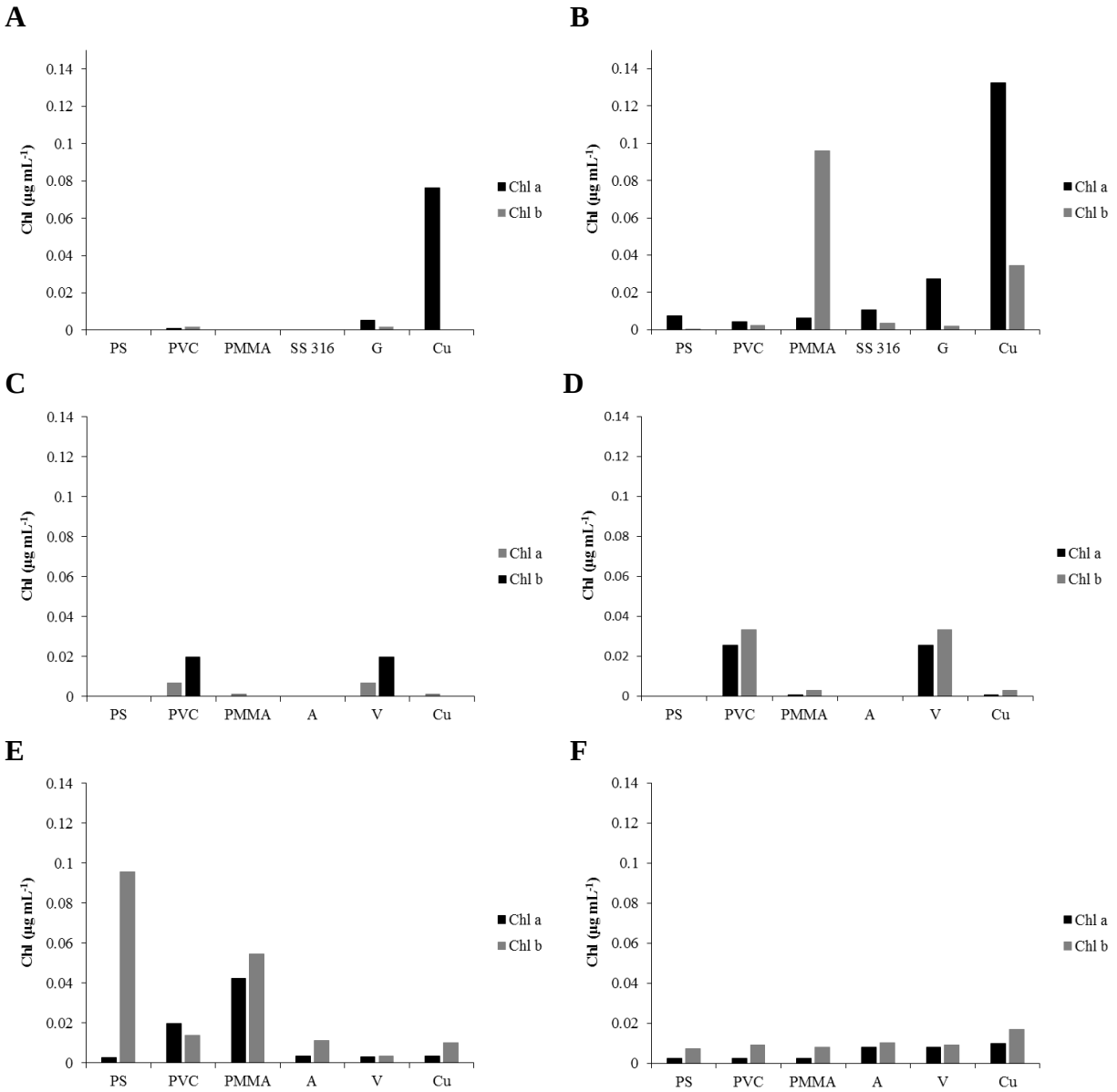

Figure A.1. Chlorophyll a and b content (µg mL^{-1}) of *C. vulgaris* (A and B), *S. salina* (C and D) and *M. aeruginosa* (E and F) adhered to the studied surfaces when using ES (A, C and E) and OECD test medium (B, D and F) as culture medium.

A.4. Microscope images

In this annexe are presented microscope images of *C. vulgaris* attached on SS 316 coupons using OECD test medium, as an example of microalgal/cyanobacterial arrangement during biofilm formation. Other images were taken for each microorganism with each surface and each culture media. However, due to the high number of images, an example is given in Figure A.2.

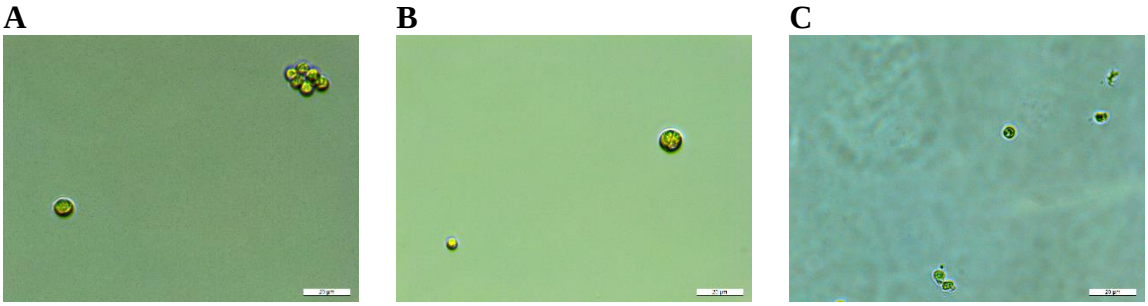

Figure A.2. Microscopic photographs of the microalga *C. vulgaris* attached to SS 316 coupons on OECD test medium at 24 h (A), 72 h (B) and 168 h (C), taken from a Nikon H550S microscope (Nikon, Japan) incorporated with a MC120 HD camera and the acquisition software LAS 4.5.0. Photographs were obtained using a 40× objective (scale bar=20 µm).

A.5. Calibration curve for nitrate quantification

Calibration curve of absorbance at 220 nm versus nitrate concentration in mg L^{-1} is represented in Figure A.3.

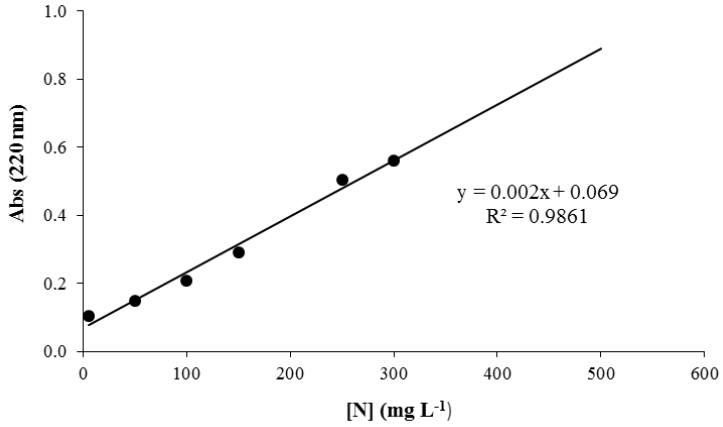

Figure A.3. Calibration curve of absorbance measured at 220 nm as a function of nitrate concentration, in mg L^{-1}.

A.6. Calibration curve for phosphate quantification

Calibration curve of absorbance at 820 nm versus phosphate concentration in mg L^{-1} is represented in Figure A.4.

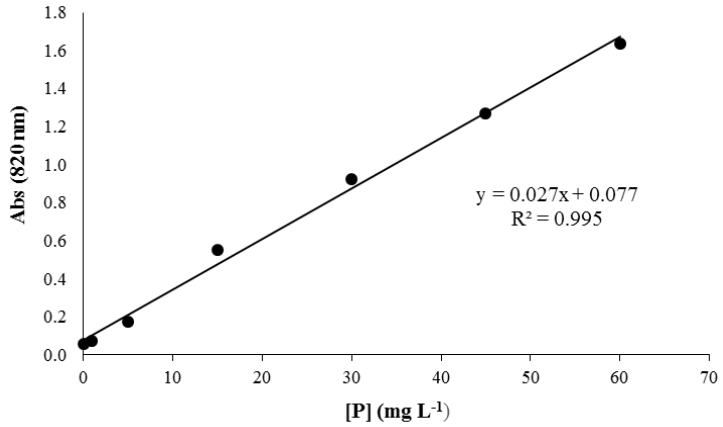

Figure A.4. Calibration curve of absorbance measured at 820 nm as a function of phosphate concentration, in mg L^{-1}.

A.7. EDS analysis of biofilm

The EDS analysis was applied to the microalgal biofilm formed on the surface of a SS 316 coupon. The results in terms of elements presented on the biofilm can be observed in Figure A.*5*.

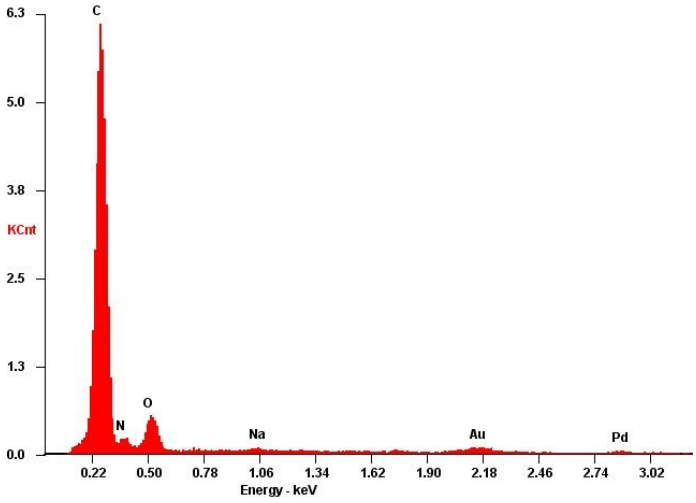

Figure A.5. EDS elemental analysis of biofilm formed on the surface of a SS 316 coupon.

A.8. OD (750 nm) profile during nutrients removal experiments

The OD at 750 nm assessed during nutrients removal using the RDR at different stirring speeds is presented in Figure A.6.

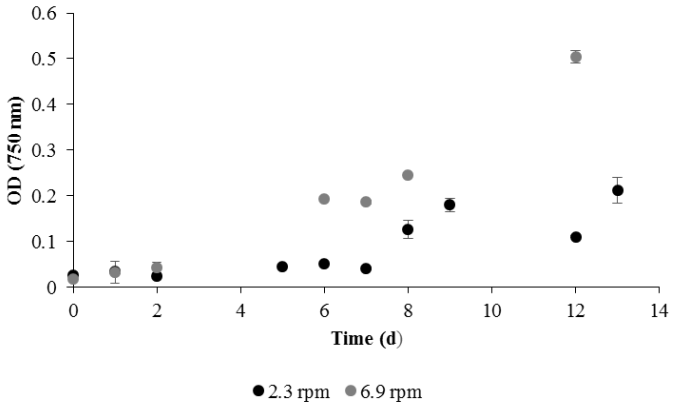

Figure A.6. OD profile of *C. vulgaris* cultured in the RDR under different stirring speeds (2.3 and 6.9 rpm).

A.9. Biofilm reactors

Figure A.7 presents the biofilm reactors, STR and RDR, in the beginning and at the end of the experiments, evidencing biofilm dynamics within the cultivation time.

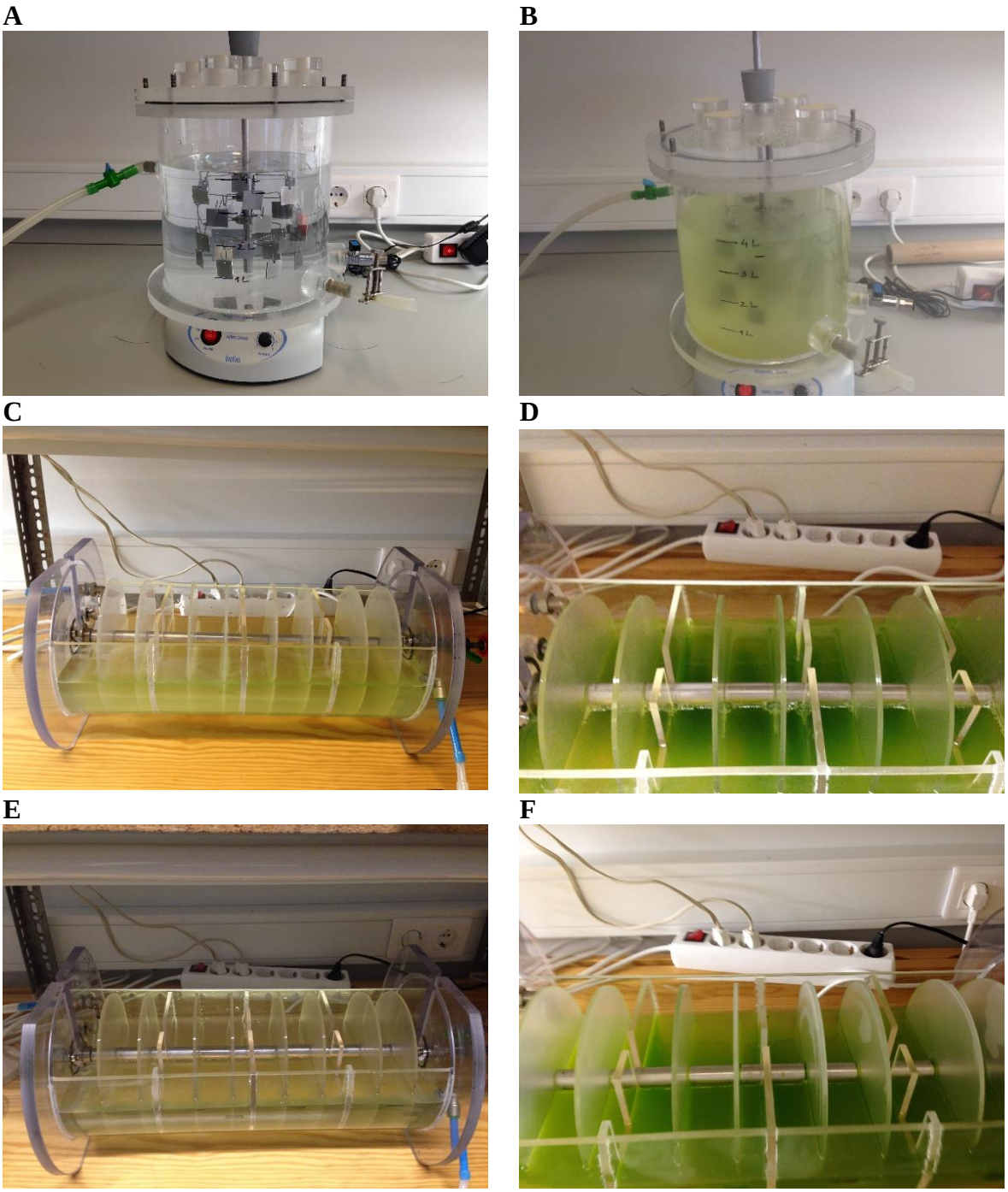

Figure A.7. Photographs of the STR (A and B), RDR at 2.3 rpm (C and D) and RDR at 6.9 rpm (E and F). The figures from the left correspond to the beginning of the experiment (day 0), while figures from the right show the biofilm formed obtained 13 days.

A.10. Microscope images of the microalgal biofilm

The microalgal biofilm formed on the bottom of the RDR was scrapped and observed on the microscope (Figure A.8).

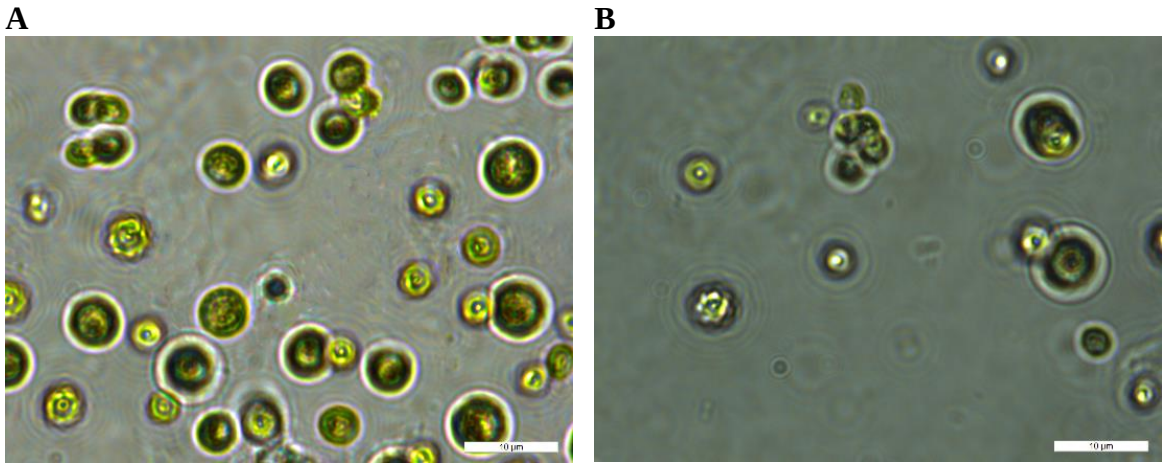

Figure A.8. Microscopic photographs of the *C. vulgaris* biofilm formed on the bottom of the RDR operating at 2.3 rpm (A) and 6.9 rpm (B), taken from a Nikon H550S microscope (Nikon, Japan) incorporated with a MC120 HD camera and the acquisition software LAS 4.5.0. Photographs were obtained using a 100× objective (scale bar=10 µm).