CRANFIELD UNIVERSITY

KATARZYNA LEDWOCH

Aeroterrestrial and freshwater microalgae biofilms: deposition and growth in aqueous and non-aqueous systems

SCHOOL OF WATER, ENERGY AND ENVIRONMENT
Cranfield Water Science Institute

PhD
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This thesis is submitted in partial fulfilment of the requirements for the degree of PhD

ABSTRACT

Non-suspended microalgal cultivation methods have gained an interest over the last decade. In contrast to traditional cultivation systems, where microalgae are grown in highly diluted suspensions, microalgae grow concentrated in biofilms over a particular substrate. Growth in biofilms gives higher biomass concentrations of end products and decreases overall water and energy consumption.

However, there are research gaps in the field of biofilm formation and growth. The studies on material and strain properties and their effects on microbial attachment are very limited. So far, a small number of strains and materials have been tested, leading to many contradictory conclusions. In this thesis the primary colonisation of 36 material-strain pairings was tested and related to topographical and physicochemical properties of substrates. Experimental data was also confronted against properties of microalgal strains. Further microalgal biofilm development in aerial conditions, and its relation to substrate properties, was analysed for the first time.

To address some of the sustainability issues associated with microalgal cultivation, a novel Humid Biofilm-Based Reactor (HBBR) was also proposed. This novel method focused on growing microalgae in a humid atmosphere enriched with nutrients. The natural phenomenon of biofilm development in aerial humid conditions was a working principle of the system, resulting in higher biomass concentrations than in other non-suspended reactors proposed so far. Using mist instead of a liquid medium significantly minimised the water consumption. No presence of a liquid medium in the reactor enabled easier maintenance of the system and improved light distribution. Growth trial in this novel reactor and its comparison to reference systems showed that HBBR was a promising way of culturing microalgae with higher growth rates, lower water and nutrient consumption, more effective light distribution and easier maintenance of the system.

Keywords:

non-suspended, cultivation, biofilm, microalgae, attachment, EPS, SMP, work of attachment, average surface roughness, hydrophobicity, wastewater, medium

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LIST OF ABBREVIATIONS

AOM	Algal organic matter
ATP	Adenosine triphosphate
BAP	Biomass associated products
BB	Bead blasted
BBM	Bold basal medium
BEPS	Bound extracellular polymeric substances
BOD	Biological oxygen demand
CA	Contact angle
CCAP	Culture Collection of Algae and Protozoa
COD	Chemical oxygen demand
DLVO	Derjaguin-Landau-Verwey-Overbeek
EBT	Enclosed biofilm tubular reactor
ECAP	Equal channel angular pressing
EOM	Extracellular organic matter
EPS	Extracellular polymeric substances
FAME	Fatty acid methyl esters
FEG SEM	Field emission guns scanning electron microscopy
FPP	Flat plate photobioreactor
HBBR	Humid biofilm bioreactor
IOM	Intracellular organic matter
MBB	Moving-bed biofilm reactor
MMA	Mycosporine - like amino acids
OWRK	Owens, Wendt, Rabel and Kaelble method
PAR	Photosynthetically active radiation
PBB	Bristles photobioreactor
PBC	Bubble column photobioreactor
PBE	Population balance equation
PDMSe	Poly-dimethyl siloxane elastomer
PETG	Polyethylene terephthalate glycol-modified
PMMA	Polymethyl metacrylate
PP	Polypropylene
PPMB	Parallel plate microalgae biofilm reactor

PRBC	Photo-rotating biological contractor
PS	Polystyrene
PVC	Polyvinyl chloride
SBBR	Submerged biofilm-bioreactor
SE	Secondary electrons
SEM	Scanning electron microscopy
SEPS	Soluble extracellular polymeric substances
SFE	Surface free energy
SMP	Soluble microbial products
SP	Sand polished
SPBR	Suspended photobioreactor
SS	Stainless steel
TD	Thermodynamic model
TEM	Transmission electron microscopy
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorous
TPP	Tubular packed photobioreactor
TSS	Total suspended solids
UAP	Utilisation associated products
UVA	Ultraviolet A
UVB	Ultraviolet B
VSB	Vertical submerged biofilm reactor
WBBR	Wastewater biofilm-bioreactor
WoA	Work of attachment
WoD	Work of detachment
XDLVO	Extended Derjaguin-Landau-Verwey-Overbeek

NOMENCLATURE

ΔG_{adh}	work of adhesion/attachment
A	area
D	diameter/ diffusivity
d_f	degrees of freedom
F	flux
K_S	growth affinity constant
L	thickness
N	number of variables
P_c	Pearson correlation coefficient
r	growth rate
R_a	average roughness
S	concentration of nutrient
t	time / two-tailed t-test value
V	volume
W_{rf}	Wenzel roughness factor
Y	yield coefficient
z	distance
α	significance level
δ	thickness
γ_c	surface free energy of cell
γ^d	dispersive component of surface free energy

γ_l	surface free energy of liquid
γ^p	polar component of surface free energy
γ_s	surface free energy of substrate
γ^{tot}	total surface free energy
θ	contact angle
θ_D	diiodomethane contact angle
θ_E	ethylene glycol contact angle
θ_W	water contact angle
μ	specific growth rate
μ_{max}	maximum specific growth rate
ρ	biofilm/culture density

1 Introduction

1.1 Project background

Demand for sustainable and feasible microalgae production method had increased over the last decade [1]. These small, unicellular microorganisms have a great role and potential in many industrial sectors: food, pharmaceutical, nutraceutical, petrochemical and fishing. Microalgal oil can be used as a feedstock in biofuel production, while many other components like proteins, polysaccharides, antioxidants, fatty acids or pigments can be utilised in commercial products [2] [3] [4].

Notwithstanding the increased research interest and application, production of low-value compounds derived from microalgal biomass, such as biofuels, is still not economically feasible. Probably the main bottleneck is the expensive dewatering step. In suspended cultivation only 1-5% of the culture volume consists of biomass [5]. Large volumes of water need to be removed in order to obtain concentrated cells. Such approach is viable for high-value algal derivatives such as compounds applied in pharmaceutical or nutraceutical industry; for example astaxanthin or β-carotene [6]. Products of significant market value allow utilisation of more expensive and less economically feasible cultivation methods. However, in the case of other algal derivatives, such as lipids, the process optimisation is essential to design a feasible production approach.

Non-suspended methods of cultivation have shown an increasingly growing potential for high-density cell culture. Unlike in traditional suspended growth, up to 20% of non-suspended culture consists of biomass [7], so product isolation requires less or no dewatering. Most of the studies on non-suspended growth focus on microalgal cells immobilised inside a matrix, such as alginate, even if retrieval of microalgal biomass from encapsulation is not an easy and cheap process. However, immobilised cells are mostly used in wastewater treatment systems, where nutrient remediation is a primary concern and microalgal biomass is just a by-product. A non-suspended alternative to encapsulation are

biofilm-based cultivation systems, in which biomass production is the main purpose. Biofilm-based systems also do not require expensive cells retrieval and generate higher products yields than encapsulated methods [8].

Biofilms can cause weathering, bio-corrosion and decolourisation of deposited surfaces [9] [10] and to date, research on microalgal biofilm is mostly limited to its negative effects. Most studies concentrate on biofilm prevention such as antifouling coatings, production of biocides [11] or design of antimicrobial structured surfaces [12]. Very little is known about microalgal biofilm formation and growth, with the majority of the studies focusing on bacterial biofilms. In addition, a better understanding of the impact of algal strains and material properties on processes governing initial adhesion and growth of cells would help in improving the design of biofilm-based cultivation systems.

1.1.1 Microalgae biomass production in non-suspended systems

Biofilm-based cultivation is the most efficient non-suspended system to biomass production [8]. In this system, microalgae are inoculated and grown on carrier materials, forming a bonded group of organisms irreversibly adhered to a substrate. This group called a biofilm is a complex community of microalgal cells characterised by individual chemico-biological characteristics and growth mechanism. Growth in a biofilm is highly beneficial for microalgae due to increased resistance to stress and hostile conditions [13], more efficient nutrient transportation [14] and better cell to cell communication [15]. Therefore, growth rates in a sessile state are usually higher than in a planktonic state [16] [17].

Various designs of biofilm-based systems have been proposed so far, although all reactors share similar cultivation principles. Microalgal cells are deposited on an attachment carrier placed vertically [18], horizontally [19] or at an angle [20]. Similarly to those in suspended systems, nutrients are delivered in the form of liquid medium and in some studies additional CO_2 is also supplied [21]. Maximum biomass productivities and concentrations in those systems are significantly higher than in traditional cultivation methods. It is possible to produce up to 40.8 grams of biomass in one square meter of attachment material per day [22]. Biomass concentration of product obtained in biofilm

bioreactors is also significantly higher: 143-192 grams of biomass per litre of water [7].

1.1.2 Biofilm development and surfaces

Understanding conditions affecting biofilm formation and development is crucial for non-suspended cultivation. Deposition and growth of biofilm is dependent on many factors [23] [24] [25] [26], probably two of the most important being the physico-chemical and topographical properties of material [27] [28] [29] [30] [31]. Many materials have been tested in biofilm bioreactors as attachment substrates such as fabrics [21] [32], polymers [33], composite materials [20], metals and glass [31] [34]. However, studies on a material's properties in relation to microalgal biofilm colonisation and development are very limited. Researchers have tested a small number of attachment materials, which often results in contradictory statements. Some studies suggest that the average surface roughness is an important parameter in microbial deposition [35]; others state it is rather surface topology that matters [36]. In some studies surface hydrophobicity was said to strongly influence the attachment [37], whereas in others no link between surface hydrophobicity and cellular adhesion was discovered [38]. Moreover, most of the studies focus on bacterial biofilms and investigate initial colonisation only. There are no studies on the relation between microalgal biofilms development and the material's properties.

1.1.3 Biofilm harvesting systems

In traditional microalgal cultivation methods, harvesting is one of the most expensive post-treatment steps, impacting the process feasibility [39]. Biomass concentration of microalgae produced via those methods is very low; 0.5 g of biomass per litre of water is usually achieved in open ponds [40]. Biomass content can be slightly higher in closed photobioreactors, reaching up to 10 g/L [41], although cultivation in closed systems is more expensive. Biomass after cultivation requires further post-processing due to the very high water to biomass ratio; it needs to be separated from the culture medium [42]. Separation is usually performed in two steps: thickening reduces water content to 93-98% and further dewatering reduces water content to 75-85% [43]. There

are many thickening techniques available: flocculation [44], gravity sedimentation, flotation [45], electrical based methods [46] and bio-flocculation [47]. Dewatering can be performed by filtration [48] and centrifugation [49]. Harvesting methods are expensive (filtration, electrical-based methods, centrifugation, flocculation), time-consuming (sedimentation), energy-consuming (centrifugation, electrical-based processes) and may involve chemical flocculants (flotation, chemical coagulation) [42]. Harvesting is the main bottleneck for the feasibility of traditional microalgal cultivation methods [43].

Biofilm-based cultivation uses different harvesting technologies. Microalgae grown in sessile state are accumulated over certain area. Because of that, it is much easier to harvest microalgae biofilms by simple scraping. Many biofilm-based systems used simple scraper to retrieve biomass from the reactor [7] [18] [19] [20] [21] [22] [34] [32]. Scraping biomass combines the advantages of suspended harvesting techniques, while practically having no drawbacks. This method is cheap, fast, simple, non-toxic, and does not require energy or the addition of chemicals, and is applicable to every microbial specie.

1.2 Project development

A detailed literature review on non-suspended microalgal cultivation methods was used to identify knowledge gaps and innovative growing methods. Working principles of biofilm formation and factors determining primary attachment were researched and summarised. Studies on aerial biofilm bioreactors were also analysed. In this context two main research gaps were identified: (1) influence of material and strain properties on attachment and biofilm development and (2) principles of biofilm development in aerial conditions.

Different surfaces and algae species were assessed to identify the best combination for optimal growth. A study on biofilm formation showed the importance of algal organic matter (AOM) excreted by microalgae: soluble microbial products (SMP) and extracellular polymeric substances (EPS). SMP accumulate on material surface and prepares the substrate for attachment. EPS bond microalgal cells together and maintain the biofilm community. The

amounts of AOM excreted by growing biofilm were also related to the removal times and work of detachment, to show the impact of SMP and EPS concentrations on overall biofilm strength. Moreover, prediction tools for cells attachment and growth were investigated and compared to experimental data. The simple Monod model showed good agreement between biofilm growth prediction and experimental data in the initial stage of development.

In parallel, aeroterrestrial and freshwater microalgal aerial biofilm growth was investigated in a humid atmosphere enriched in nutrients. Finally growth of *Chlorella saccharophila* in the novel Humidity Biofilm-Bioreactor (HBBR) was compared to growth in liquid in suspended and non-suspended conditions.

1.3 Aims and objectives

The main aim of this thesis was to analyse the factors influencing microalgal biofilm formation and development and to determine the overall viability of biofilm-based cultivation, with focus on non-aqueous systems.

To do so, the following objectives needed to be fulfilled:

- To produce a state of the art review on aerial-non-suspended microalgal growth;
- To evaluate and compare primary attachment of aeroterrestrial and freshwater strains on different materials;
- To identify and appraise the factors affecting primary adhesion and biofilm development;
- To design and develop of a novel non-aqueous biofilm-based growth system;
- To estimate non-aqueous biofilm-based growth of different microalgae species;
- To optimise the non-aqueous biofilm bioreactor.

These objectives have been summarised in the general overview of the thesis presented in Figure 1:

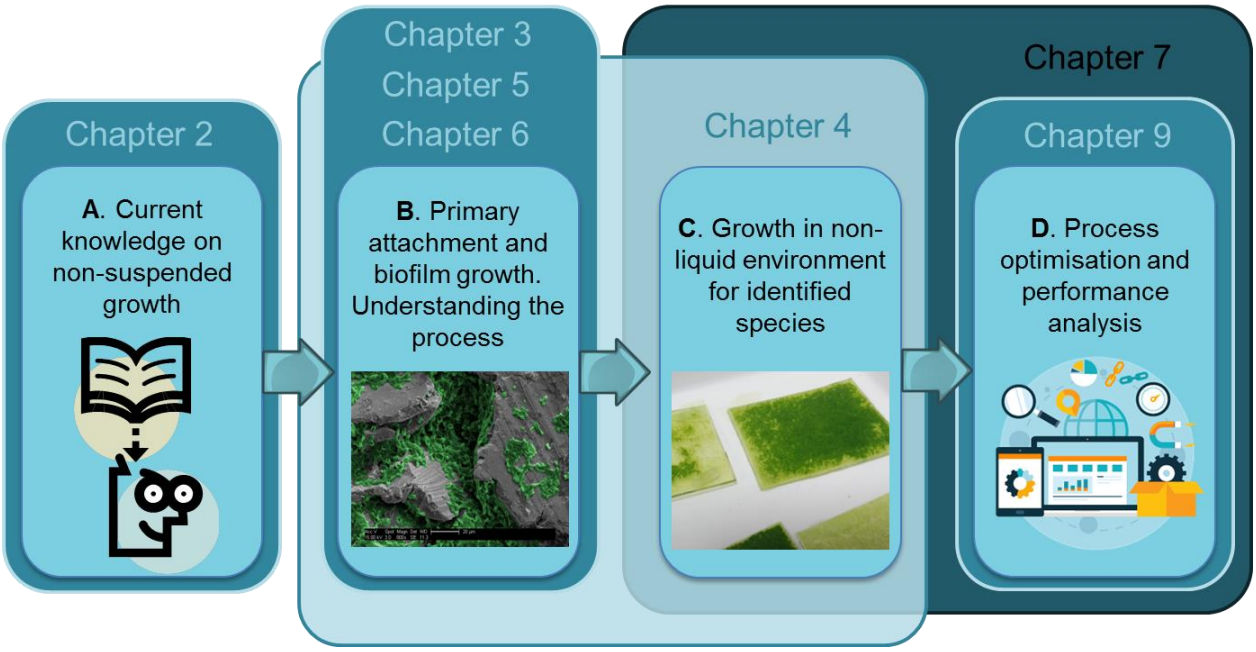


Figure 1 Graphic summary of the thesis objectives

1.4 Thesis outline

This thesis is presented as a series of chapters formatted as papers for publication. All papers were written by Katarzyna Ledwoch, and edited by Dr Raffaella Villa (principal supervisor) and Prof Bruce Jefferson. All experimental work was designed and performed by Katarzyna Ledwoch at Cranfield University (UK). Relations between chapters are presented in Figure 2. Each chapter was represented by different colour set.

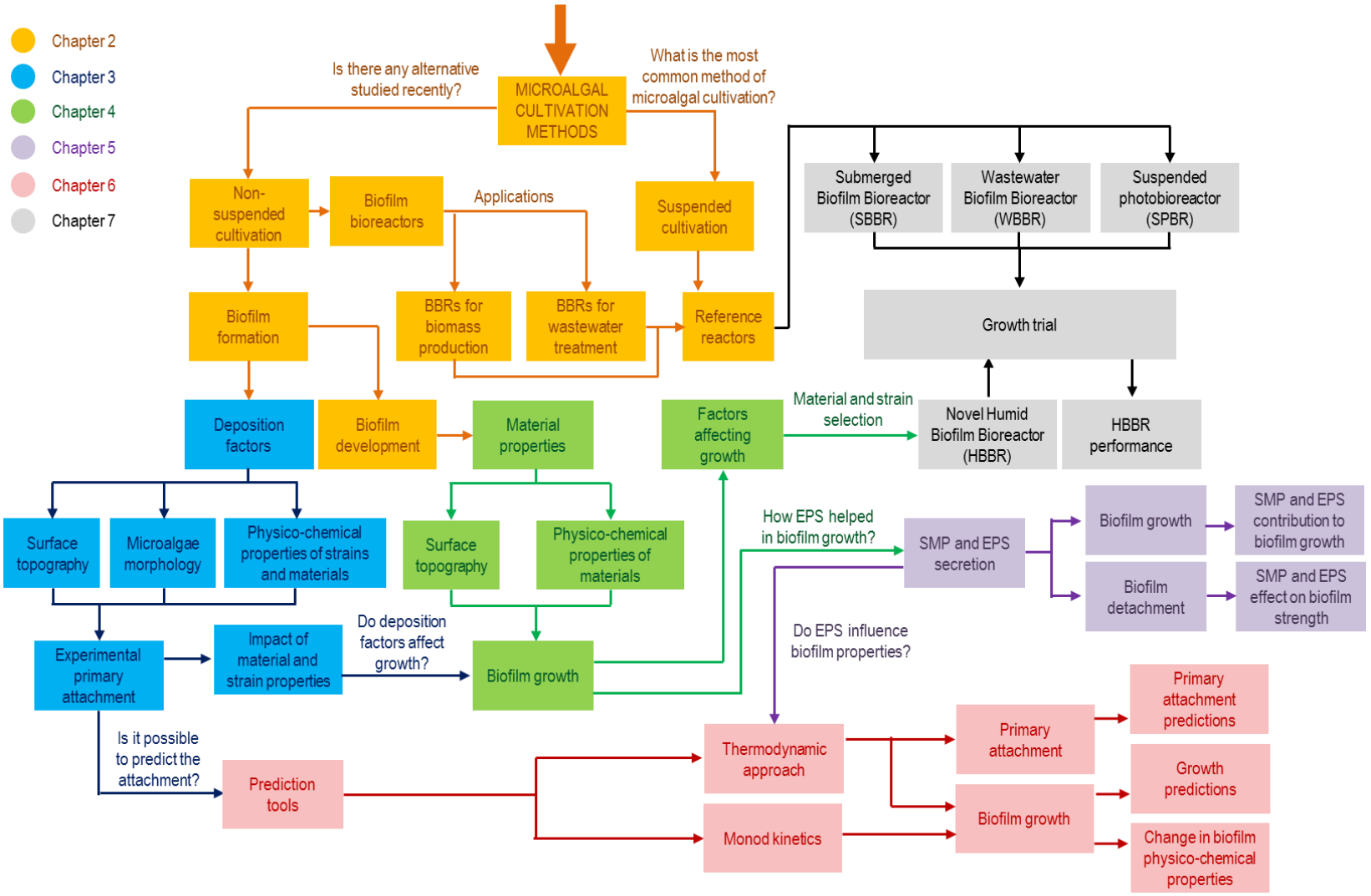


Figure 2 Thesis overview

1.4.1 Chapter 2 Literature review and research needs

Chapter 2, 'Non-enclosure methods for non-suspended microalgae cultivation: literature review and research needs', focuses on microalgae suspended and non-suspended cultivation methods. This chapter provides an overview of available cultivation techniques, their advantages and issues. This chapter was published in the 42th volume of *Renewable and Sustainable Energy Reviews* in 2015 [50]. Figure 3 shows the structure of the literature review.

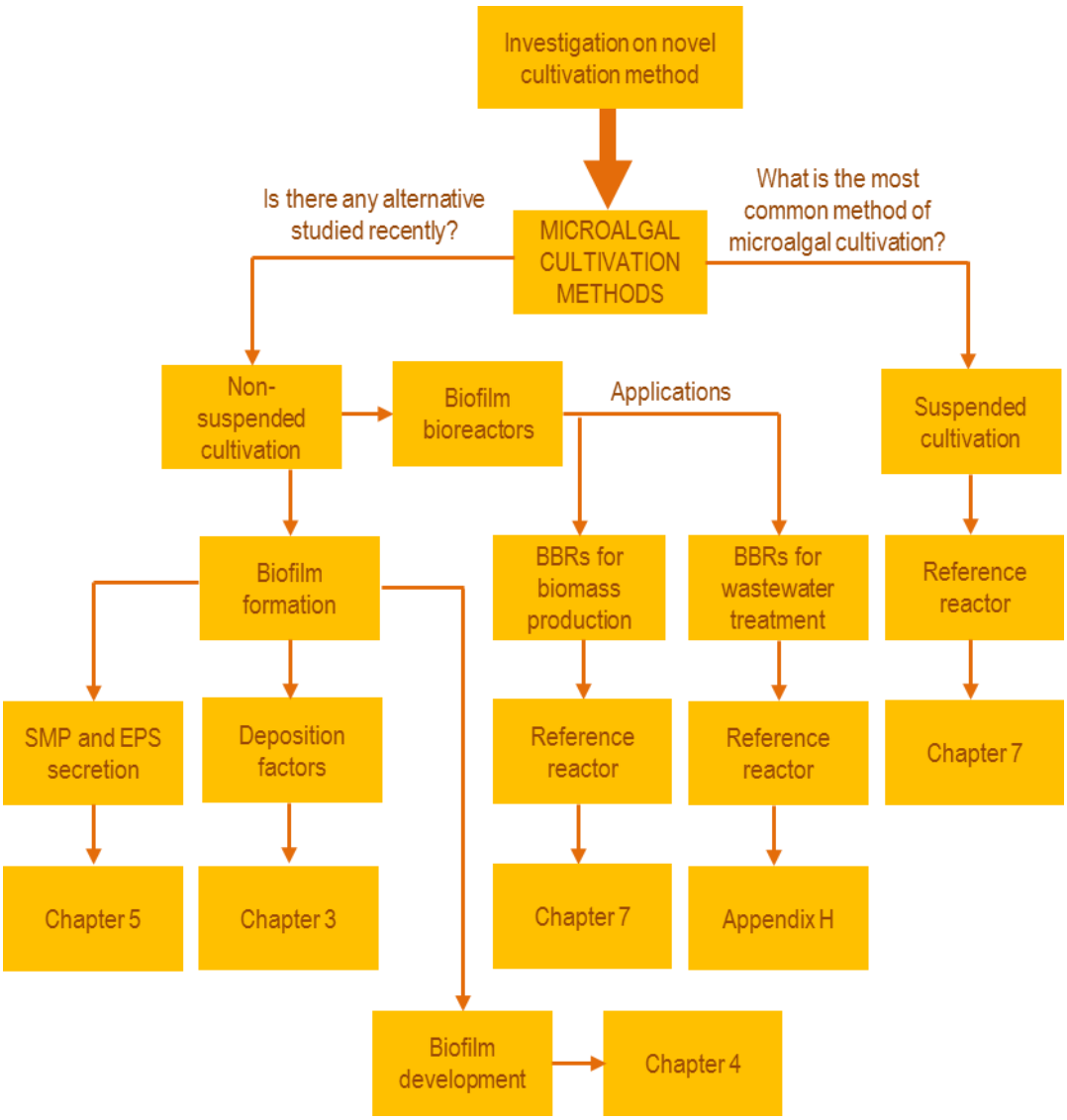


Figure 3 Chapter 2 overview

Factors affecting the biofilm attachment and growth are also discussed, to underline existing research gaps in the field of microalgal biofilm deposition and development.

1.4.2 Chapter 3 Impact of strains and materials' properties on primary attachment of aeroterrestrial and freshwater microalgae

Chapter 3 main purpose was to determine which properties of strains and substrates affect biofilm deposition (Figure 4).

Although many factors influencing the colonisation of surfaces were analysed by other scientists, there is still a considerable gap in the field of microalgal biofilm formation and growth. Processes of initial attachment and biofilm development are still not fully understood [51] [52] [53]. The impact of physico-chemical and topographical properties of materials on biofilm formation and development has been so far researched on a limited number of strains and materials, mostly comprising bacteria. It is therefore very hard to establish what properties of materials influence the attachment. A higher variety of materials and species would need to be tested to address the attachment phenomenon.

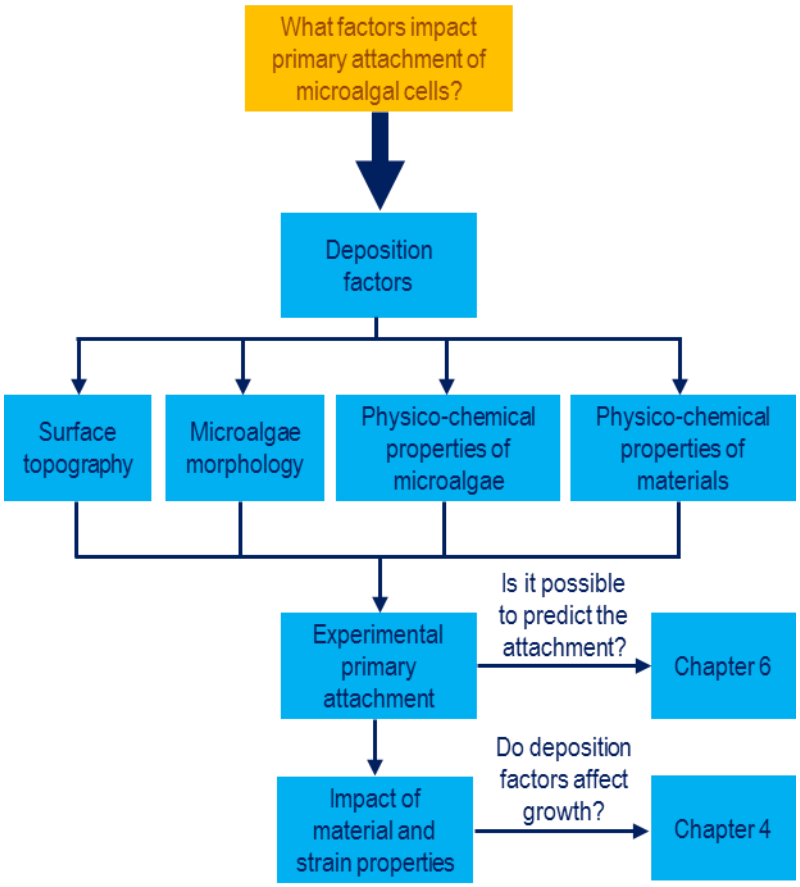


Figure 4 Chapter 3 overview

In this study, 36 microalgal-material pairings were investigated. The aeroterrestrial species *Chlorella luteoviridis* and *Stichococcus* sp. are microalgae naturally isolated from biofilms on building facades, rocks or trees. The two freshwater species were *Chlorella saccharophila* and *Haematococcus pluvialis.* 9 materials of different types: steel, plastics and glass were investigated. Primary attachment of *C. saccharophila, C. luteoviridis, Stichococcus* sp. and *H. pluvialis* was analysed in relation to physico-chemical properties of attachment carriers and strains. Impact of material topography and cell morphology was also investigated.

1.4.3 Chapter 4 Impact of material properties on freshwater and aeroterrestrial biofilm development in aerial conditions

Primary attachment is an important step in biofilm formation as it determines the number of cells that will grow into the biofilm. However, knowledge on the

microalgal biofilm development process in relation to substrate properties is still very limited. Most of the work has been done on bacterial biofilms and tested on a small number of materials. Moreover, to the best of the author's knowledge, there are no such studies on microalgal biofilms in aerial conditions.

Chapter 4 focuses on the influence of material properties on biofilm development (Figure 5). Microalgal strains were grown in a humid atmosphere for 5 days, in order to mimic the natural conditions for aeroterrestrial species growth. In total, 24 different microalgal-material pairings were analysed.

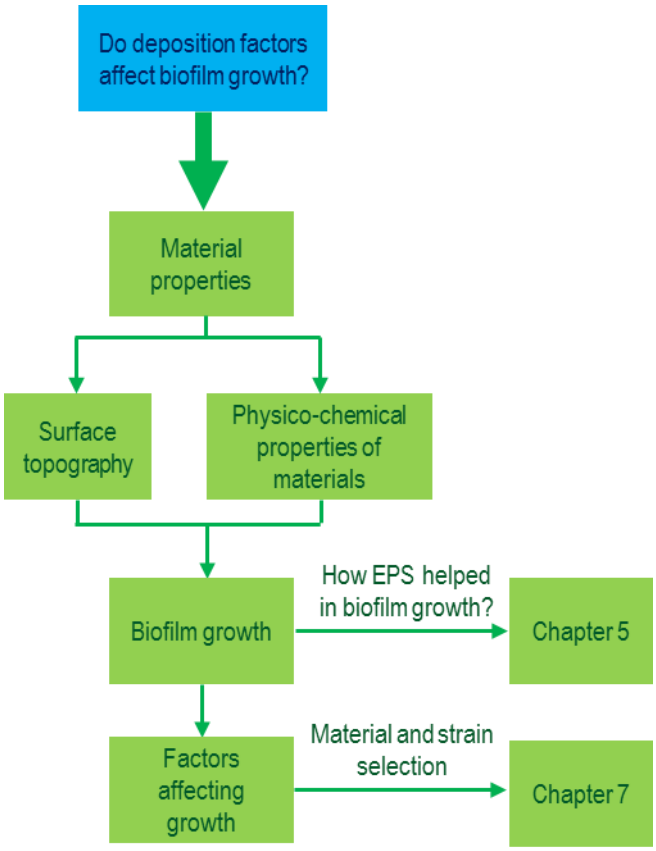


Figure 5 Chapter 4 overview

Growth of 24 material-strain pairings helped in selection of the final material and strain that was used in growth trial in Chapter 7. The choice was made based on the experiments performed in Chapter 3 and 4.

1.4.4 Chapter 5 Role of cellular EPS, SMP and surface topography of attachment substrate on biofilm development and strength

Chapter 5 shows the importance of AOM excretion in biofilm formation and growth (Figure 6). EPS and SMP were identified as important components in biofilm formation.

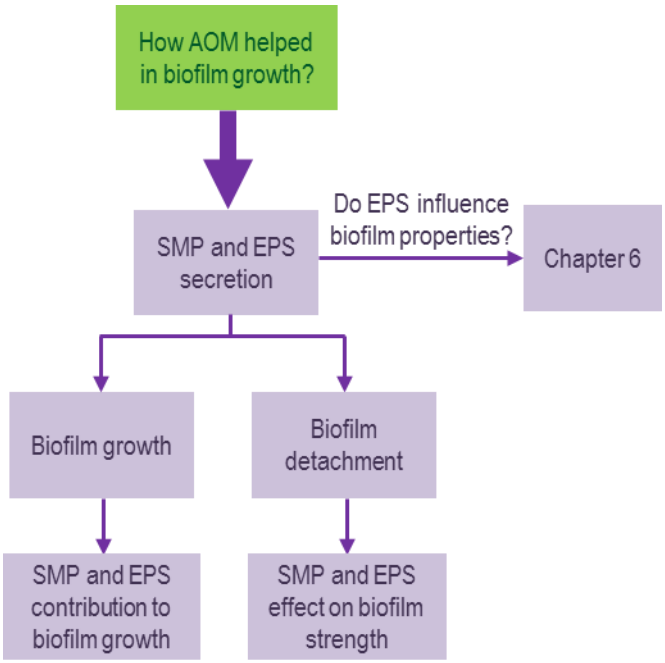


Figure 6 Chapter 5 overview

SMP's influence on microalgal attachment was not clear; vast majority of studies on SMP excretion focused on their contribution to membrane fouling. Chapter 5 helps in understanding the essential function played by SMP in microalgal attachment.

Further study on EPS secretion and their influence on growth on rough and smooth substrates was performed. Rough polystyrene (PS) and smooth polyethylene terephthalate (PETG) were chosen as attachment carriers. Polyol-free *Stichococcus* sp. and ribitol-containing *C. saccharophila* were tested. The study from Chapter 5 helped in determining how microalgae maintained high growth rates on smooth materials and how lack of polyol affected EPS secretion for *Stichococcus* sp.

To test whether the AOM emission strengthens the biofilm, secretion of SMP and EPS was compared to the energy required to detach biofilm. There was no such study on the influence of AOM excretion on biofilm strength conducted before.

1.4.5 Chapter 6 Prediction tools for attachment and biofilm growth

Many experimental tests needed to be performed in order to understand the mechanisms governing biofilm attachment and growth. It was also very time consuming to select the best microalgae and material among initially selected strain-substrate pairings. Therefore, the simplest tools for primary attachment and biofilm development predictions were analysed in Chapter 6 (Figure 7).

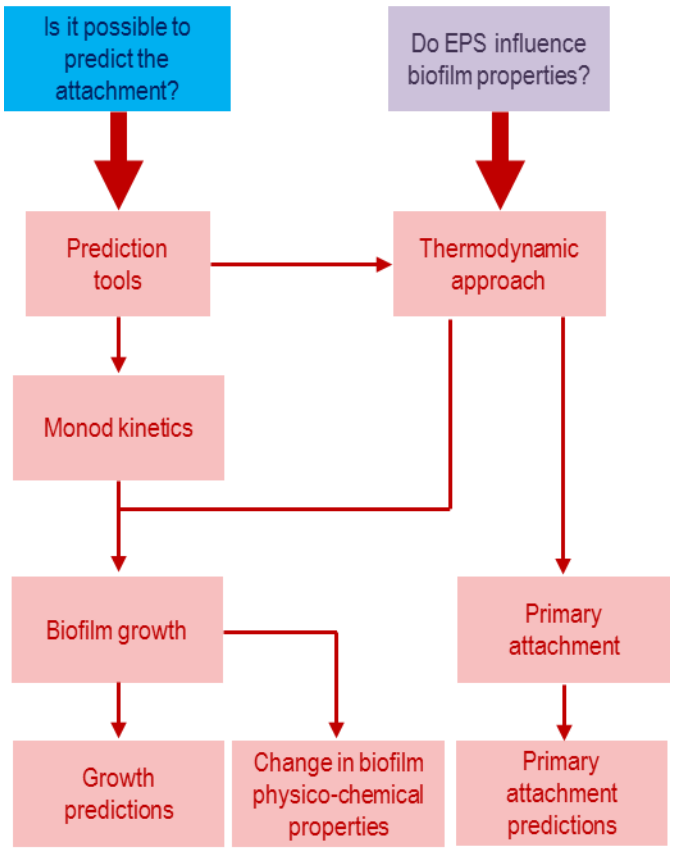


Figure 7 Chapter 6 overview

The thermodynamic model was used to predict primary attachment of 4 species and 9 materials to show whether there is a 'shortcut' for material-strain pairing selection.To investigate applicability of simple growth predictions model in

biofilm-based systems design, Monod kinetics [58] was utilised to describe development of *Stichococcus* sp. and *Ch. saccharophila* microalgae on PS and PETG substrates. Both models were confronted with experimental data on adhesion and growth of microalgae on substrates.

1.4.6 Chapter 7 Non-suspended aeroterrestrial cultivation of *Chlorella saccharophila* in humid atmosphere: novel Humid Biofilm-Bioreactor (HBBR)

Non-suspended reactors gained popularity as they produce biomass with higher cell concentration [16] [17]. Therefore, the whole process of biomass production is cheaper, as the dewatering step could be omitted (for some applications) or significantly reduced. However, the majority of non-suspended cultivation methods still utilise a high volume of water, as microalgae are grown in biofilms inside liquid medium. Chapter 7 (Figure 8) include the data related to the novel microalgal growth in a humid atmosphere. The novel cultivation reactor was tested with the best strain and substrate, selected based on previous investigations described in Chapters 3 and 4. HBBR performance test was conducted for 42 days and the growth inside reactor was compared with non-suspended and suspended reference reactors.

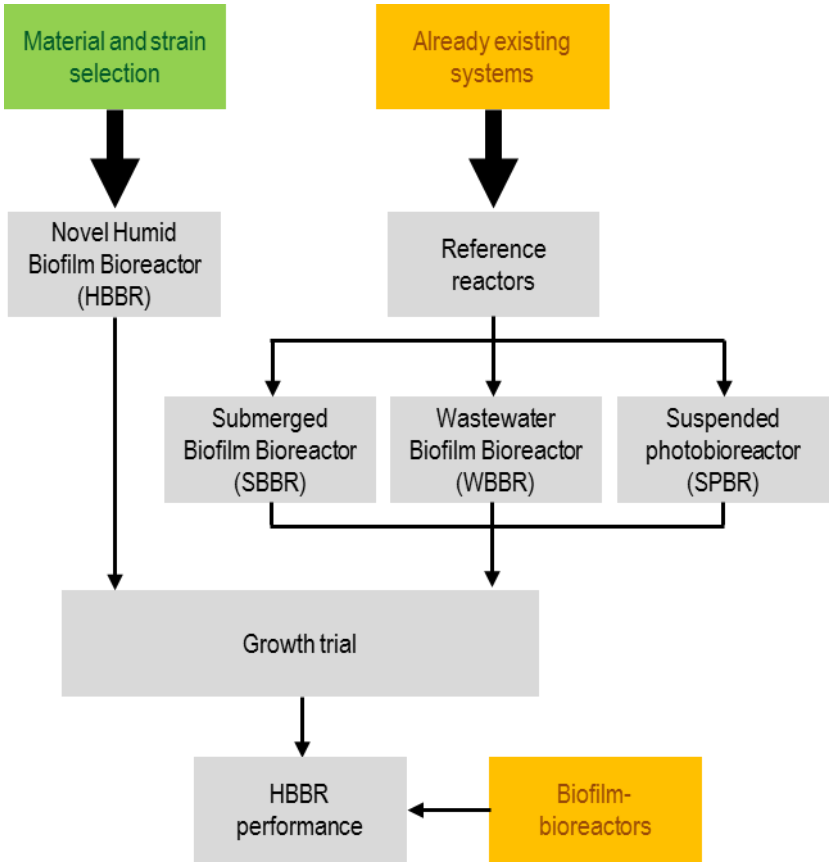


Figure 8 Chapter 7 overview

1.4.7 Chapter 8 Overall implications for biofilm growth in humid environment

Chapter 8 summarises the most important conclusions made based on work conducted in this thesis. Mechanisms controlling algal biofilm formation and development are discussed. The viability and potential business case of HBBR are given.

2 Non-enclosure methods for non-suspended microalgae cultivation: literature review and research needs

2.1 Abstract

Microalgae are receiving more attention both from industry and science communities. There are countless applications of these unicellular microorganisms from fourth generation biofuels, to food and feed, to pharmaceuticals. Traditional methods of cultivation are associated with many problems such as high costs, high energy consumption, and low product yields. Scientists are investigating alternative methods of microalgae cultivation and processing to overcome these problems. One of the newest approaches is the non-suspended method for microalgae culturing, where microalgae are grown attached to surfaces.

Growing microalgae on surfaces is an attractive option and shows promising results. In comparison to ordinary suspended photobioreactors, the attached systems offer higher biomass yields, easier scale-up, better light distribution within the reactor and better control of contamination. The consumption of water can also be reduced using this method. Using aeroterrestrial algae in attached cultivation systems, in humid aerial conditions that would mimic their natural growth environment, could potentially decrease water consumption to a minimum. In humid conditions, a biomass of lower water content can potentially be produced, even in comparison to existing biofilm-based systems. Furthermore, the mechanization of the cultivation and harvesting processes would be less complex in humid conditions, as the product would not be immersed in liquid. To evaluate the potential advantages of non-suspended culturing of aeroterrestrial microalgae in a non-enclosure method, proper experiments need to be conducted. In this review, basic concepts of attached cultivation systems are discussed, focusing on the studies of biofilm formation and including factors affecting deposition. Finally, the detailed description of

aeroterrestrial microalgae is included to give an insight into potential applications of the species into attached cultivation systems.

2.2 Introduction

The interest in microalgae has drastically increased over the last few decades, given their appealing applications in many areas, from fish food, to new generation biofuels, to pharmaceuticals; including specialized medicines, cosmetics, fertilizers, and many more [59] [60].

Scientists have been working to make the production of algae more commercially viable. However, there are many challenges, and harvesting is one of them [39]. Processing large volume of microalgae culture is expensive and time-consuming. One possibility is to accumulate microalgae on surfaces during cultivation to allow easy collection. To date, non-enclosure microalgae cultivation has not yet received enough attention. There is no reported study on non-suspended cultivation of aeroterrestrial microalgae. Most of the aeroterrestrial microalgae researchers focus on the problems caused by microalgal biofilm formation, by analysing mechanisms of attachment and anti-fouling methods of growth control and prevention. This review is to discuss the potentials of using aeroterrestrial microalgae in non-enclosure microalgae cultivation. Literature on the microalgae biofilm formation on artificial substrate is investigated to establish future research routes for microalgae cultivation.

2.3 Microalgae cultivation: an overview

2.3.1 Suspended vs non-suspended cultivation

The most common approach in algae cultivation is the suspended method, where microalgae are grown suspended in the medium. Algae cells flow freely inside the container and additional mixing is required to ensure the even distribution of cells. This method results in a low concentration of algae grown. The dilution of microalgae in suspended systems is high. Around 99% of culture volume consists of water [5] and only the remaining 1% is the dry algal biomass. To obtain a dense product, huge amounts of biomass need to be processed. Therefore, harvesting large volume of microalgae is an extremely

expensive process [61]. Supplying water to maintain microalgae production is also of high importance. It is projected that about 3800 kg of water is required to obtain 1 kg of biodiesel [62]. Therefore, a huge amount of water is needed for processing microalgal growth in suspended cultivation systems. The productivity in current suspended systems is low [16]. So far, a maximum of a few grams of dry biomass per litre of media can be produced during one day of suspended cultivation [63] [64]. The productivity depends on various factors such as microalgae species, reactors and culture density. In the table below there are some selected examples of biomass productivities (Table 1). In non-suspended mode, algae are grown on surfaces. It leads to the accumulation of dense algae inside the reactor. They can be enclosed in the matrix (enclosure method) or form a biofilm on the surface (non-enclosure method) [33]. With the non-suspended way of cultivation, it is much easier to separate microalgae biomass from the medium, as significant quantities of biomass are accumulated on small area [65]. For harvesting, algae are scraped and dried in the case of non-enclosure method. For enclosure method, a post-processing step is required to extract microalgae from the matrix.

Table 1 Biomass productivities for different kind of suspended cultivations

Algae specie	Productivity [mg/L per day]	Type of cultivation	Reference
***Chlorella* sp.**	3200	Closed	[66]
	4025	Open	[67]
Chlorella vulgaris	40	Closed	[68]
	136	Mixotrophic	[69]
Spirulina platensis	320	Open	[70]
	320	Mixotrophic	[71]
	2100	Closed	[70]
Botryococcus braunii	26	Closed	[72]
	155	Closed	[73]
Scenedesmus obliquus	140	Closed	[74]
	150	Closed	[73]
Haematococcus pluvialis	76	Open	[75]
	410	Closed	[76]

The non-suspended method can be more commercially feasible than ordinary suspended microalgae cultivation. In attached cultivation systems, microalgae are placed on a substratum with water supplied only to keep the surfaces wet.

Such system was introduced by Liu *et al.* in 2013, reaching an average productivity of 70.9 g/m^2 per day for *Scenedesmus obliquus* [16]. In the same reactor, *Botryococcus braunii* reached productivity of 5.5 g/m^2 per day [17]. The production of biomass is given in grams per square meters in those tests. Both results were compared with ordinary suspended cultivation (Table 2).

Table 2 Productivity comparison of suspended and non-suspended cultivation [17]

Species	Attached cultivation productivity [g/m^2 per day]	Suspended cultivation productivity [g/m^2 per day]	Reference
Scenedesmus obliquus	70.9	8.9-14	[16]
Botryococcus braunii	5.5-5.7	2.4	[16] [17]

Costs associated with water consumption are lower in non-suspended cultivation. To manufacture one tonne of microalgae in an attached cultivation method, only 17 tons of water are needed for circulation with four tons consumed for surfaces to sustain an appropriate wetness level [16], whereas in suspended cultivation around 220 tons of water is consumed [62]. Untill now, there is no attached system operating on a big scale for biofuel production, nevertheless promising results are obtained from laboratory scale experiments [32] [7].

2.3.2 Enclosure vs non-enclosure methods

Non-suspended cultivation can be generally divided into enclosure (encapsulation) and non-enclosure approaches. Both methods rely upon the cultivation of microalgae in biofilms. In enclosure methods special gelling agents are used to immobilise the cell, whereas in non-enclosure methods cells are allowed to grow freely in a naturally formed biofilm. The interest in the enclosure method of non-suspended microalgae cultivation is growing, as microalgae are easier to control when encapsulated inside the matrix [8]. Experiments on this particular method of cells immobilization are straightforward, given the well-established techniques used for enzymes and organelles entrapment [77] [78]. However, to separate algae from the matrix is not an easy task [33]. The

compounds of enclosure may have effects on microalgae species and scaling-up the process would be expensive [79].

In non-encapsulating methods, the strain is grown on artificial substrate placed inside a liquid medium. The main advantage of non-enclosure methods over encapsulation methods is simplicity of operation. Biofilm formation process occurs naturally when substrate is brought to contact with microbial culture. Cells have a natural tendency to form biofilm in water habitats, therefore simple inoculation step is enough to allow biofilm deposition. Wild biofilms of different microalgae species can be found commonly. By creating a biofilm, it is easier for cells to maintain and protect themselves from biocides, predators, and medium conditions (such as pH or temperature).

Non-enclosure approach does not require addition of gelling compounds. Moreover, to obtain microalgal biomass no retrieval from the matrix is required. In the result, non-enclosure methods are more cost effective for microalgal biomass production than encapsulation methods.

Many microalgal species grow on surfaces [16]. They are found on ships, inside reactor tanks or even on building facades in the natural environment [23] [80]. Biofilms are rich in different species of microorganisms, such as bacteria, fungi, or microalgae [81]. Similar depositions also take place in human organisms, such as blood platelets or dental plaque [82].

Some studies were performed to investigate non-encapsulated growth of microalgae [83], although the number of available data in regard to the positive aspect of biofilm formation is limited. Nevertheless, there are many studies on biofouling that are also relevant in this field of study [84]. Naturally occurring biological layers have no direct benefits. There are many examples of the negative influence of biofilm creation [23], such as the pollution of drinking water, reduction of thermal performance for boilers, and possible toxins generated by some algae species [85]. Scientists have been researching biofouling control and prevention, using biocides, reversal flow, ultraviolet light, and anti-fouling coatings [23].

2.3.3 Aquatic vs aeroterrestrial microalgae

Microalgal selection is a crucial aspect in the design of new approaches in cultivation. The growth of cells is strongly strain-dependent; two various microalgal species could grow in a complete different manner in the same conditions. Microalgae are unicellular microorganisms, widely appearing in aquatic and terrestrial environments. They play a very important role in ecosystems. There is a large number of microalgae species with 30,000 species discovered and an estimate of possible a 70,000 species [86]. Microalgae can be divided according to their taxonomic group or living environment. There are many known microalgal strains, mostly known for their valuable by-products such as lipids or antioxidants. However, in the aspect of non-suspended cultivation it is important to choose a microalgae strain that not only will be able to create a biofilm, but also grow well in the community. The vast majority of microbes have a natural tendency to create a biofilm, although the quality of the biofilm formed varies between species. Microalgae that are able to excrete natural cell-bonding components and form well-structured aggregates would be the best option for non-suspended cultivation.

The most commonly researched species are saltwater and freshwater microalgae, generally referred as aquatic species. Examples of those strains are *Chlorella vulgaris, Scenedesmus obliquus*, *Pyrocystis lunula*, or *Nannochloropsis oculata*. Aquatic microalgae are known to naturally form biofilms in water environment, colonising ships or reactor walls. In most cases aquatic microalgae contribute to biofouling and are seen as detrimental phenomenon. However, some of the species were proven to effectively grow in non-suspended reactors, resulting in higher biomass yields in comparison to traditional suspended cultivation. Aquatic microalgae, like terrestrial ones, are also responsible for surface degradation. They form an unwanted biofilm on ships and industrial tanks. Their biofilms can be found on any artificial surface that is immersed in natural water reservoir for a longer period of time [23].

Aquatic microalgae are appropriate species for suspended and non-suspended cultivation, as both methods rely on cultivation of species inside a liquid

medium. However, if a different way of cultivation is to be considered, the selection of species would need to be based on special characteristics of a novel design. For example, the method of growing microalgae in a humid atmosphere would require a special kind of microalgae that is able to form biofilms in more hostile conditions that aquatic microalgae. Aeroterrestrial microalgae are species growing in biofilms, colonizing both natural and artificial surfaces. They can be found on roof tiles, statues, building facilities, damp rooms, rocks, trees, soil, and many more non-aquatic environments of high humidity [9] [87] [88]. They may be found growing together with bacteria, fungi, protozoa, and cyanobacteria [9] [89]. Biofilm thickness can reach up to 0.1 millimeters. Example strains are *Klebsormidium* sp., *Stichococcus* sp., *Coccomyxa sp*., and *Apatococcus* sp. [80] [87] [90] [91]. Most of the terrestrial algae can be found in green algae groups, such as *Trebouxiophyceae* or *Chlorophyceae* [92]. However, aeroterrestrial microalgae diversity is not well understood [93]. Relevant research mostly focuses on their negative impacts on building facilities. Biofilms causes decolourisation and the faster weathering of surfaces they deposited on [9] due to microbial actions triggering breakdown of those materials. Their contribution to the surface weathering is significant, especially that their growth is faster than the growth of higher plants. Species such as *Gloeothece* sp., *Chlorella* sp., *Schizotrix* sp., or *Chroococcus montanus* are good examples of terrestrial microalgae that degrade surfaces [94] [95].

Aeroterrestrial microalgae have a unique ability to survive desiccation for a long period [13]. They can even survive in a drought when the reproduction is stopped. In comparison with aquatic cultures, aeroterrestrial microalgae are flexible and shrink during dry periods (even to around 60%) [96], which is highly beneficial in biofilm reactors of varying environmental conditions. Aquatic microalgae such as *Nannochloropsis* sp. and *Scenedesmus dimorphus* do not withstand desiccation well. After drying their growth is significantly limited [97]. That is why aquatic species could not grow well in aerial or humid conditions. By contrast, aeroterrestrial microalgae are easily revived after preservation by drying. It takes only few minutes for *Stichococcus* sp. and *Chlorella luteoviridis* to recover photosynthesis after moisturizing [80]. They are highly resistant to

hostile environmental conditions. As a result, variations in salinity, temperature, and UV do not affect them as much as marine algae [90], which makes aeroterrestrial microalgae a perfect material for open biofilm bioreactors situated at uncontrolled conditions. Aeroterrestrial microalgae have a high survival rate at extremely low temperatures. Most of 27 aeroterrestrial species tested by Lukesova *et al.* in 2008 survived cryopreservation, with survival rates above 50% in most cases [98]. There were even species exhibiting 100% of survival rate: all cells of *Cylindrocystis brebissoni* and *Chlorella fusca* species survived conservation in extremely low temperature (almost -200°C). That makes aeroterrestrial species more resistant than aquatic cultures.

2.4 Biofilm formation

The formation of a biofilm is a crucial first step in non-suspended cultivation. Quality and quantity of attachment determine the further development of the biofilm and impact the final products yields. Biofilms occurring in natural environment are generally created by bacteria, larvae, fungus, protozoa and microalgae [81] [99]. They can be found even in extreme and unfriendly environments, such as nuclear power plants or hydrothermal vents [100]. The formation of a microalgae layer is a complex process [23] while the adhesion mechanism is not fully understood [101] [27]. It is believed that hydrophobic reactions are driving forces for biofilm formation on hard substrates [82].

The first and probably the most important step is the creation of a conditioning film [23] [102]. This is a base layer on the surface for microorganisms to grow on. There is no clear evidence that the conditioning film is required to create a biofilm [103], however its formation is essential in promoting cells deposition [104] [105]. Conditioning film formation takes place straight after the surface is immersed into the medium, creating the layer consisting of ions and organic molecules [102]. Once the conditioning film is formed, microorganisms start their attachment. In the case of saltwater, it takes a few hours for microalgae to attach to the surface [106].

Further growth of the biofilm involves the reproduction of microorganisms by division, rather than absorbing free floating particles from the surrounding

medium [23]. Before reaching the exponential growth phase, microalgae undergo a lag phase (time needed to start reproduction) [107]. In the first few days of mixed biofilm formation, microalgae are the microorganisms that dominate the biofilm composition [108], then diatoms start to take over and eventually cyanobacteria become dominant [81]. In most biofilm bioreactors axenic cultures are cultivated and efforts are put on contamination prevention. Co-aggregation of other species is often seen as detrimental phenomenon, especially when the system aim is the production of a certain compound that is secreted by a particular microalgal strain.

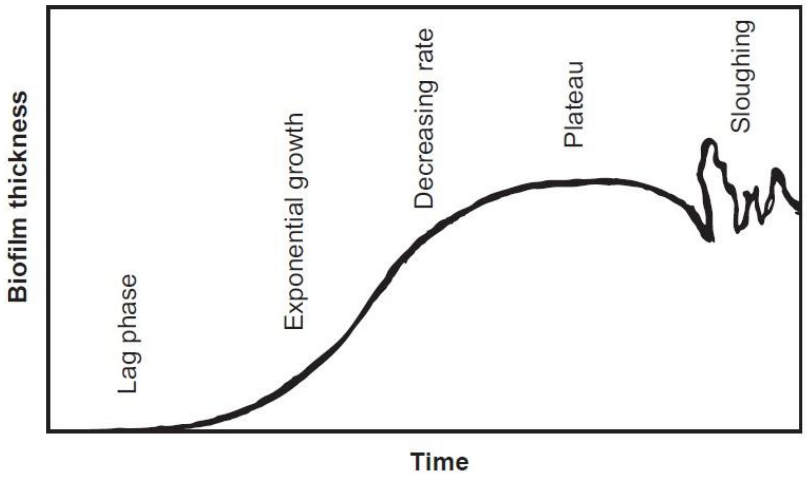


Figure 9 Growth of biofilm in time [23]

The growth curve of microalgae in a biofilm is similar to that of aquatic algae (Figure 9). The lag phase is followed by exponential growth, then the rapid development of biofilm stops and reaches the maximum biofilm thickness, at the end the mature biofilm undergoes sloughing.

2.4.1 Algal organic matter

Algal organic matter (AOM) is a compound secreted by microalgae during biomass growth. AOM is formed by soluble microbial products (SMP) and extracellular polymeric substances (EPS). AOM is widely known for its negative impacts on the environment, for example in the drinking water industry [109]. However, for biofilm deposition and development, the presence of SMP and EPS is essential.

2.4.1.1 Soluble microbial products (SMP)

SMP are organic compounds secreted by microalgae during cellular growth and decay [110]. They can easily accumulate on the substrate surface and penetrate through cavities [111], as they are soluble in liquid medium [112] [113] [114]. However, their function in primary attachment of cells and further biofilm development is still not clear.

2.4.1.2 Extracellular Polymeric Substances (EPS)

Most microalgal species secrete polymeric substances that help in formation and development of biofilm matrix. During biofilm formation, cells produce EPS [115] to create a matrix bonding the whole biofilm together [23] [24]. This creates the environment for the growth and reproduction of microorganisms and enables the easy attachment of external particles [116]. EPS consists of various groups that function as metal-binding sites. Examples could be negatively charged carboxyl or phosphate group or polysaccharides, proteins, nucleic acids, lipids, phospholipids, and humic substances [117] [118].

EPS plays an important role in nutrient exchange. They are also responsible for cohesion (binding cells together) and adhesion (binding cells and substratum) [119]. EPS not only acts as a nutrient sink, but also protects the whole structure of the biofilm from grazing [120] [121] and action of harmful biocides [122].

Aeroterrestrial microalgae also produce EPS. EPS excreted by terrestrial algae play the same role as in the case of aquatic microalgae, although some additional benefits have been discovered. In addition to the functions mentioned above, EPS protects aeroterrestrial algae from desiccation, retaining water inside the algal cells [89] to enable a longer survival during drought. It helps in the survival of terrestrial species such as *Chlorella trebouxioides, Chlorella luteoviridis*, or *Stichococcus bacillaris* [89] [95].

2.4.2 Deposition factors

Various factors contribute to the growth of biofilm with nutrients and light intensity generally regarded as the most important for all microalgal species [23] [24]. In the case of aeroterrestrial microalgae, the key factors for growth also

include water availability [80] [87] [93] [123], as terrestrial species are not grown in liquid and are dependent upon the relative humidity of the surroundings. The other important factor determining biofilm deposition in aerial environments is the chemical/physical properties of the surfaces [102]. The type of surface is very important, as some attachment surfaces can store the water and the storage ability increases with porous materials [123]. Aeroterrestrial algae prefer rough and porous materials [95] and smaller temperature amplitudes. It is because smaller variation in temperature and the presence of cracks can keep the surface wet and prevents or reduces water evaporation [94] [124]. Nutrient concentration is also significant in the case of aeroterrestrial microalgae, however, it does not have as much influence on the biofilm composition as other factors mentioned before [125] [126]. Properties of materials and nutrient availability are also important in aquatic microalgae attachment, however for slightly different reasons. Rough substrates provide shelter from shear forces of moving liquid [127], whereas high concentration of nutrients may result in stress response in cells. Moreover, microalgae perform a self-detachment process, when nutrient availability is too low.

Other factors affecting the adhesive strength, amount of biomass formed and its composition could be: disturbance [128], surface roughness [31] [33] [39], pH [31], surface rugosity [55], irradiance [25], fluid velocity [26], and concentration of free-floating cells in the medium [26] [129]. More details about their influences on biofilms will be discussed in the following chapters.

To investigate the influence of different factors on biofilm formation, it is important to establish parameters measuring its growth including biofilm thickness, cell counts, or dry mass of formed biofilm [130]. A typical apparatus used for monitoring the biofilm is the “Robbins device” [23] [102]. In the Robbins device, test plates are placed inside an aluminium block. The liquid is passed through a flow channel and the biofilm is formed on the test plates. It is possible to remove these plates later and study the accumulation of biomass as well as the influence of liquid velocity. The other techniques for cell biomass or biofilm activity measurement are scanning electron microscopy (SEM), transmission

electron microscopy (TEM), scanning controlled laser microscopy, adenosine triphosphate (ATP), total organic carbon (TOC) measurement, light microscopy, and Confocal Scanning Laser Microscopy [23] [102] [130] [131].

All the factors mentioned above have an influence on the development of microalgal biofilm and its composition, however the extent to which they are affected mostly depends on the strain to be attached [33].

2.4.2.1 Light intensity

The availability of light determines the presence of microalgae in naturally occurring biofilms [23]. The intensity can increase or decrease their adhesion. The attachment is weaker with limited light and generally microalgae growth increases with light intensity [24]. Once the growth reaches its limit, cells undergo photoinhibition and the growth declines.

Light intensity influences aeroterrestrial microalgae in a different manner than aquatic microalgae. The study on *Stichococcus* sp. and *Chlorella luteovirdis* species [83] showed that aeroterrestrial microalgae exhibit high tolerance to ultraviolet A (UVA) and ultraviolet B (UVB) radiation. It is due to the presence of mycosporine-like amino acids (MMA), absent in *Ulvophyceae* or *Chlorophyceae* group. In contrast, aquatic alga *Desmodesmus subspicatus* was affected by too high irradiation by slowing its pace of growth. Some of aquatic microalgae species can stop their growth in the presence of UVA and UVB radiation (Table 3) [83]. The great tolerance of aeroterrestrial microalgae to variations of light intensity is very advantageous, as they are less prone to photoinhibition [132].

Table 3 The effect of photosynthetically active radiation (PAR), UVA and UVB radiation on selected algal species [83]

Conditions						
Photosynthetically active radiation (PAR)					**50 PPF**	
Ultraviolet radiation (UV)					**8 W/m^2 UVA 0.4 W/m^2 UVB**	
Specie	Type	MMA	Growth in PAR+ UVA	Growth in PAR+UVB	Recovery in PAR+UVA	Recovery in PAR+UVA/ B
Stichococcus sp.	Aeroterrestrial	Yes	No change	No change	Full	Full
Chlorella luteoviridis	Aeroterrestrial	Yes	No change	No change	Full	Full

Myrmecia incise	Aeroterrestrial	Yes	30% decline	43% decline	Full	Full
Desmodesmus subspicatus	Aquatic	No	33% decline	Inhibition	Full	80%

2.4.2.2 Nutrient concentration

Nutrients are essential for the development of microalgae film. The amount of nutrients should be maintained at a suitable level. Above that level, the attachment of cells stops increasing [104] [133]. In mature biofilms, the cells closest to the substratum surface have limited access to nutrients [23]. It results in sloughing of whole biofilm. The need for appropriate nitrogen, phosphorous and other elements strongly depends on microalgae strain. For example, some species require extra amount of silica [134]. Addition of glucose to biofilms can enhance the accumulation, however, the structure formed is loose [26].

Aeroterrestrial microalgae are more capable to withstand hostile conditions than freshwater species. Terrestrial species had to adapt to the low concentration of nutrients on aerial substrates they colonise. The preference towards nutrient rich surfaces was observed [9], although the ability of aeroterrestrial species to survive at nutrient deficient environment is beneficial in open biofilm bioreactors. It also makes them more resistant to nutrient drop across biofilm layer. The composition of nutrients needed by aeroterrestrial microalgae is unique for each species, similarly as in the case of aquatic microalgae.

2.4.2.3 pH

The pH of the cultivation medium affects microalgal growth and biofilm establishment [135]. The structure is influenced by pH even more than by nutrients [90]. It can also happen that the pH within microalgal layer is different from the surrounding medium [26] when microorganisms create a whole new environment separated from the surroundings during biofilm formation. The best attachment of *Nitzschia amphibian* to titanium and glass was obtained approximately at neutral pH environment [31]. This is within the acceptable range given for most algae species grow well at pH levels from 7 to 9 [136]. The exceptions are green algae found in soils, which prefer acidic conditions [137].

2.4.2.4 Flow of medium

Movement of surrounding medium influences the biofilm thickness [23] and could be one of the most important factors affecting adhesive strength [26]. Laminar flow, occurring at low velocities, generates a thick laminar sub-layer, which enables material to accumulate in a dispersed manner [23] and make it easy to remove cells. When the fluid velocity increases, the mass transfer between particles floating in the medium and biofilm increases as well. In the range of 0.6 to 1.6 m/s of fluid velocity, the strength of attachment is improved for *Pseudomonas fluorescens* [138]. However, the removal of cells from the existing biofilm is also enhanced. An optimal flow velocity could be found to achieve the maximum growth of the microorganism's layer (Figure 10) [138]. Aeroterrestrial microalgae do not grow within the fluid and the movement of fluid hardly affects them. The presence of water in the form such as rain, highly humid air, fog, or snow is more important [80]. Natural tendency of aeroterrestrial species to grow at humid atmosphere may be highly beneficial in novel, currently investigated non-enclosure cultivation at aerial humid conditions. Terrestrial species are capable of utilising moisture from humid air, which makes them a good candidate to be grown outside liquid medium.

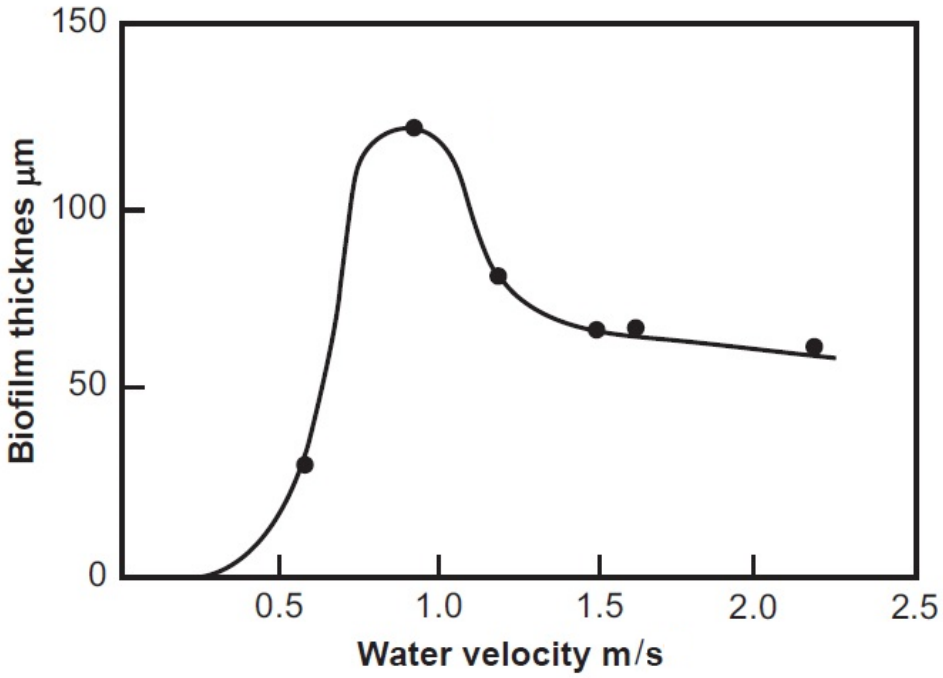


Figure 10 Dependence of biofilm thickness on water velocity [138]

2.4.2.5 Microalgal selection for products recovery

Non-suspended systems rely on a natural tendency of microalgae to form biofilms. Not all species are capable to establish complex communities, therefore in a biofilm-based system design it is important to select microalgal strain able to grow at sessile state.

Even though the ability of microalgae to create biofilms is an essential criterion in specie selection, algal species well growing in community might not be the best for product recovery. In order to maximise products yields and values, it is essential to identify other characteristics of microalgae that need to be taken into account at specie selection stage. Ability to withstand desiccation periods is essential in biofilm growth [89]. Periodical decrease in water availability has a detrimental effect on some algal strains growing in biofilms [13]. When cells are not able to recover after drought, decrease in growth yields is often observed, which impacts the overall productivity of biofilm-based system. It is important to select microalgal species that could resume the growth as quickly as water is available. Moreover, selected specie should be able to secrete compounds (such as lipids, proteins or carotenes) under stress conditions. For example, *H. pluvialis* is able to effectively accumulate astaxanthin even when grown in deficient environment [76].

To minimise the impact of environmental effects on microalgal growth and thus maximise products recovery, it is essential to select species that have similar or higher production yields in sessile state in comparison to their planktonic state. It was reported that some species secrete compounds and grow at higher rates when in community, such as *Scenedesmus obliquus* or *Botryococcus braunii* [16] [17].

Some microalgal species may interact with attachment substrate, for example inducing corrosion on steel surfaces [139]. Wear of substrate may have a detrimental effect on overall system performance. Therefore, selection process should be performed based on possible interactions between adhesion surface

and microalgae strain. For example, when steel materials are selected as adhesion substrate, it would be better to select *Porphyridium purpureum* rather than *Oscillatoria* sp. [140].

Microalgae species have different characteristics and behaviour. To give insight into differences between microalgal strains, examples are presented below:

– Preferences in the way of cultivation: It is evident that some strains may prefer to grow on surfaces while others grow more favourably within the medium as shown by the comparison between Bristles Photobioreactor (PBB) and Bubble Column Photobioreactor (PBC) [28]. It is found that *Amphora* sp., *Navicula* sp., and *Nitzschia ovalis* strains preferred attachment on surfaces with the best results in terms of concentration and biomass yield [28] in PBB (non-suspended cultivation). In contrast, *Nitzschia* sp. and *Cylindrotheca closterium* grew better inside ordinary PBC (suspended cultivation).

– Preferences in medium properties: It is reported that *Chlorella vulgaris* forms thicker biofilm on unsterilized medium and no such observation was found in the case of *Scenedesmus obliquus* [29].

– Different predispositions to create biofilms: It was found that not all algal species are capable of producing EPS which affects the quality of biofilms created [29]. An example of such microalgae is *Chlorella vulgaris*, unable to produce EPS by itself.

– Different influence on attachment surface: For non-suspended culturing, microalgae species may have direct influence on the attachment surfaces. It is found that some microalgae acts as precursors in microbiologically induced corrosion [139] due to the change in pH value and oxygen release during biofilm formation. However, not all algal species induce the biocorrosion of the substrates. It is reported that *Porphyridium purpureum* does not contribute to steel corrosion [141].

– Strain-specific approaches in attachment improvement: It is possible to stimulate some algal species to accumulate on surfaces. $CaCl_2$ was added to

improve *Chlorella sorokiniana* to form aggregates [142]. Growing *Chlorella vulgaris* in non-sterile water led to more cells attached to the substratum [29].

2.4.2.6 Cell size impact on primary adhesion

Literature lacks discussion on direct relation between cell size and its attachment to substrates. Studies investigate only the preference of cells towards material structural features (such as cavities, dimples) that are similar or slightly higher in size than cell size of tested specie [143] [144].

Cell size is known to influence physiology, molecular composition, metabolism and sinking rate. It is also connected to the rate of culture growth, as it impacts resources absorption, such as light, nutrients and CO_2. Size distribution of cells helps in monitoring the growth phase [145], as the processes of growth and cell division are influenced by the cell size ratio.

Growth and cell division are simultaneously occurring in the course of biofilm development. Cells of smaller equivalent diameter at the start of cultivation are able to stabilise quicker and achieve higher growth rates than cells of bigger size [145]. It could be implied that biofilms containing cells of a small diameter can develop faster. On the other hand, according to Stokes law, increasing cell radius is accompanied by increasing sinking rate [146] [147]. Hence, bigger cells should be approaching the substrate-liquid interface quicker than smaller cells. So far, it is unknown how the cell size distribution of the starting inoculum can influence primary colonisation of cells on substrates. It is possible that improved growth rates of small cells and sinking rate of big cells are not a rule applied to species in general, but to cell size within the same species.

2.4.2.7 Cell physical properties impact on primary adhesion

Cells' hydrophobicity was proven to be an essential factor in their propensity to attach [148]. It is easier for hydrophobic cells to attach to hydrophobic surfaces, presumably because they can overcome repulsive forces [149]. In addition, microorganisms are able to switch between their hydrophobicity/hydrophilicity depending on environmental changes in temperature or nutrients [150]. For example, gram-negative bacteria are able to release outer membrane vesicles

in order to increase hydrophobicity of their cells and therefore increase the predispositions to form biofilms [151]. Physical properties of cell and their impact on adhesion ability have not been broadly studied.

2.4.2.8 Substrate properties

The selection of appropriate carrier substrate is as important as the selection of microalgae strain. Characteristics of the surfaces are critical for initial biofilm formation. According to studies, following factors need to be considered in particle deposition:

– Roughness and texture of surface: They play important role in particles deposition, microalgae grow better on rough surfaces [39] [33] [31] [55] [152] [153]. The study of the red algae showed that *Halosaccion glandiforme*, attached to substrata with structural features had about 35 times larger density in comparison with density obtained on smooth surface [39]. Also the proper size of substratum dimples can elevate attachment. When dimples are slightly larger than the size of the cells to be deposited, the attachment is greater [33] [154].

– Hydrophobicity: Biofilms are created on hard substrates generally due to hydrophobic reactions [82]. To encourage microorganisms' attachment, it is preferable to have hydrophobic surfaces in particular for saltwater [155].

– Presence of protective layers on surface: Bacteria and diatoms have strong tendency to colonize surfaces, so they can be met even on specially designed antifouling coatings [156]. However, it needs to be kept in mind that microorganisms are less likely to colonize on substratum covered with hydrophilic coating [157]. It is also important to take into account the influence of attachment surface sterilization, as this process changes the properties of the surface [141].

– Costs of surface production: To make non-suspended algae cultivation feasible, the substrate materials need to be cheap and environmentally friendly [61]. Surface texturing, desirable in particle deposition enhancement, should be an efficient and not cost consuming process [61] [31].

In his work on microalgae attachment, Cui tested a great variety of substrates: teflon, polycarbonate, polypropylene nylon 6/6, glass microscope slides, and stainless steel 304 [33]. Microalgae tend to accumulate most on the material with the lowest surface free energy, nylon (34.6 ergs/cm^2). According to Cui, surface free energy had bigger impact on particle deposition than surface roughness, as stainless steel possessing the roughest surface (124 nm) from all tested materials was not attaching the highest amount of cells [33].

In another study, Sekar *et al.* [31] conducted experiments with perspex, titanium, stainless steel 316-L, glass, copper, aluminum brass, and admiralty brass. Their results showed that the highest attachment took place on stainless steel and titanium (Figure 11). The remaining materials exhibited weaker promotion of microbial adherence.

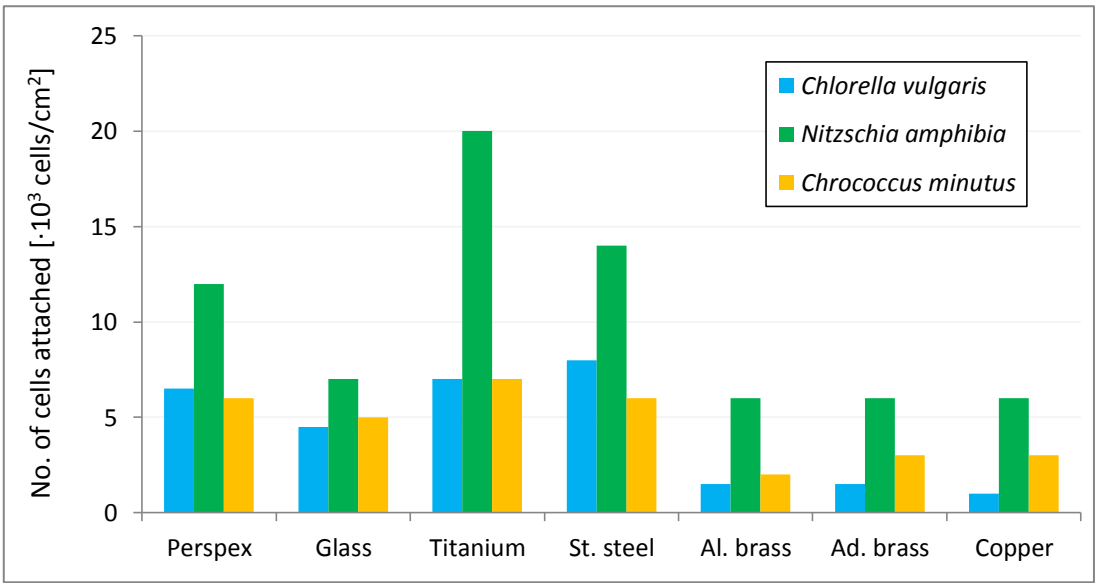


Figure 11 Growth of *Chlorella vulgaris, Nitzschia amphibia*, and *Chrococcus minutus* on different materials [31]

During studies on rotating algal biofilm system, Gross *et al.* [21] showed that the most effective material for growing microalgal biofilms for biofuel production is cotton. It was better than other tested materials, such as microfiber, fiberglass, nylon, or vermiculite. The same conclusion was made by Christenson and Sims [32]. According to their research, cotton cord was more effective than nylon, polypropylene, acrylic, or jute [32]. Other studies on microorganisms attachment

involved polydimethylsiloxane, polyimide, polycarbonate plates, silicon, alkane thiolates, plexiglas, and poly-dimethyl siloxane elastomer (PDMSe) [29] [37] [55] [84] [158] [159] [160] [161] [162] [163]. All materials above were tested regarding the mechanism of attachment, contact angle data or antifouling properties rather than information related to which material is the best for growing microalgae in biofilms.

2.4.2.9 Surface roughness impact on primary adhesion

The data reported in **Error! Reference source not found.** contains a summary of the research work reported in the literature on the relation between material's roughness and primary microbial adhesion. Only few studies were performed on microalgal biofilms whilst the vast majority of studies were conducted on bacterial biofilms, which have a higher impact on human lives such as the colonisation of medical devices in implantation or dentistry [38] [164] [165] [166] or biofouling in food and drink industries [30] [54]. Some of the anti-fouling compounds or biocides widely used for biofouling prevention are not suitable or harmful to patients. Therefore, most of the work in biofilm prevention is related to factors that affect microbial deposition but do not impact human's health such as surface roughness and material structure. Topographical modifications of carrier substrate surface allow chemical-free and antibacterial agents-free method of bacterial colonisation inhibition [12].

In the study of Sokolova *et al* [30] there was no visible relation between diatom/bacteria attachment and the roughness of xerogel coatings. Roughness data was not measured, but taken from literature values which could have influenced the uncertainty of the results [30]. Average surface roughness of materials was also not measured in the work performed by Sekar *et al* [31], Sreekumari *et al* [54] and Johnson [39]. Sekar *et al* conclusions, that increase in average surface roughness is always accompanied by increase in attachment, were not fully proven as they were made based on polishing grit size of titanium and stainless steel with only one strain, *Nitzschia amphibia*. In addition, the actual difference in materials' structure was evaluated by optical inspection and not through consistent instrumental measurements. It is important to distinguish

between reliable numerical values of average roughness (R_a) and visual structure of the surface.

Microalgae have wide cell size spectrum. Smaller pico-eukaryotes have diameters smaller than 1 µm, while the biggest dinoflagellate or diatoms can reach more than 1000 µm [146]. Nano-roughness of substrates is the most commonly investigated property of material. **Error! Reference source not found.** shows that a vast majority of studies tested samples of average surface roughness lower than 0.15 µm. Nano roughness of substrate is particularly important in dentistry [167] as oral biofilm formation is the source of problems on dental abutment, leading to localised mucositis or peri-implantitis [168].

In some studies, a high error of attachment constrained proper analysis of data [35] [55]. In the work of Cui *et al* [35], the difference in *Nannochloropsis oculata and Scenedesmus dimorphus* attachment to four stainless steels of varying roughness was not high when the error was taken into consideration. *N. oculata* adhesion on 1850SA and 1000SA steels was equal to 0.31±0.05 and 0.36±0.05 cells·10^3/mm^2, respectively. Taken into account errors of those measurements, the attachment to much rougher (R_a=57.8±0.4 nm) 1000SA steel was almost the same as to smoother (R_a=37.6±0.6 nm) 1850SA steel. Similar relations were found for *S. dimorphus*: 4.6±0.5 and 4.9±0.6·10^3 cells/mm^2 corresponded to materials with R_a equal to 37.6±0.6 and 57.8±0.4 nm, respectively. Moreover, 6.2±0.6 and 6.9±0.25·10^3 cells/mm^2 attached to 1200 SA and SS304 steel materials of R_a equal to 60.7±0.6 and 124±4.7 nm, respectively. Therefore, it was not correct to conclude that adhesion depended on surface roughness amongst materials of the same composition, as suggested by the authors. Köhler *et al* [55] results also had very high error and among 10 strains tested, only *Needle diatoms*, *Mytilus edulis* and *Polydora* sp. adhered more to rougher substrates.

In contrast, some researchers suggested negative correlation between adhesion and surface roughness. According to Bagherifard *et al* [169] and Truong *et al* [170], bacteria attach better to smoother materials. However, only one material was tested in each of these studies. In the work performed by

Bagherifard *et al*, accumulation of mixed bacteria culture on stainless steel 316 with three different roughness values was identical (~$9 \cdot 10^3$ cells/cm^2) and with a very high error, approximately equal to $2 \cdot 10^3$ cells/cm^2 [169]. In the study of Truong *et al* [170], *S.aureus* strain attached well to smoother ECAP polished titanium. Yet, another strain, *Pseudomonas aeruginosa*, attached in the same manner to both materials with biofilm evaluation error equal to 1μm for a 2μm biofilm thickness.

Singh *et al* [165] observed that the correlation between attachment and roughness might not be linear. According to their work, *Escherichia coli* and *Staphylococcus aureus* cells adhered more with increasing roughness of titanium films. However, only to a certain value; afterwards there was a visible drop in attachment. The highest attachment was obtained for titanium film of 100 nm thickness and roughness equal to 21.7 nm. However, also in this case, the experiment was based only on 4 points and the difference between roughness values was very small; 21.7±1.1 nm and 25.5±1.6 nm.

De Souza and Ferragut [56] stated that there is no correlation between polyethylene surface roughness and algal periphyton adhesion. Morgan and Wilson [57] showed that for cold-cured denatures acrylic plugs there was a relation, whereas for heat-cured not.

Finally, other studies just underlined the importance of substrate roughness in adhesion of cells [164] [166]. Most authors did not attempt to explain how exactly the roughness affected the attachment. From the data available in literature it is difficult to draw any conclusion especially because the difference in roughness between the studied substrates has often been extremely small (nanoscale). Hsu *et al* [166] did not correlate quantitative amount of *Escherichia coli, Listeria innocua* and *Pseudomonas fluorescens* attachment with surface roughness of alumina and silica but focused on the patterns formed by bacteria. Gross *et al* [36] just stressed the importance of surface texture, comparing cell attachment on smooth plastics to the attachment on plate supported mesh. Resultant mesh structure significantly increased the attachment of cells, as they

were able to utilise characteristic square pores and use them as a shelter against flow of medium.

It is possible that the scale of the surface topography plays an important role in the attachment process and that this value should be in the same range/scale of the attaching cells [38]. However, the average surface roughness expressed by scalar value should not be the only factor to consider when studying microbial cells propensity to attach. Materials with the same average value of R_a may in fact, have completely different topography. Therefore, structure of the material need to be carefully analysed, for example by scanning electron or confocal microscopy. In particular, presence of irregularities that favour attachment and decrease shear forces of flowing medium provide an attractive substrate for microbial adhesion [127] [171].

2.4.2.10 Physical surface properties impact on primary adhesion

Physical properties of attachment carrier affect propensity of cells to adhere (**Error! Reference source not found.**). One of the most important factors is hydrophobicity/hydrophilicity of material, expressed by water contact angle [155] [82] [172] [37]. Ista *et al.* [37] showed that for decreasing water contact angle of self-assembled monolayer, the attachment of *Ulva linza* and *Cobetia marina* increased. The same conclusions were made by Klein *et al.* [173], when they showed that hydrophobic substrates were preferred by *Navicula jeffreyi* diatoms.

Cui *et al.* [35], Fletcher and Pringle [174] showed that attachment increased with decreasing surface free energy of substratum. However, Fletcher and Pringle [174] work based only on three points describing polystyrene SFE vs bacterial attachment. Likewise, Cui *et al.* [35] assumption was made based on nylon, stainless steel and glass only, and just for one sample of each material. Moreover, the trend was visible in the case of *Scenedesmus dimorphus*, but there was no such phenomenon in attachment of *Nannochloropsis oculata*.

The relation between SFE and adhesion of cells does not always take place. Gross *et al.* [36] observed that there is no relation between attachment of

Chlorella vulgaris and SFE of tested materials. Only relation to tetradecane contact angle was found. Lack of correlation was also reported by Sokolova *et al.* [30] and Sousa *et al.* [38]. They concluded that surface free energy of xerogel coating [30], acrylic and silicone [38] substrates did not affect the adhesion strength.

Table 4 Surface roughness effect on primary microbial adhesion

Reference	Number of species	Types of strains	Number of materials	Types of materials	Average surface roughness of tested substrates (R_a)	Outcome
Sokolova *et al.*, 2012 [30]	3	*Cellulophaga lytica* (bacteria), *Navicula incerta* (microalgae), *Amphibalanus Amphitrite* (diatoms)	1	xerogel coatings	66 µm, 54 µm, 0.6 nm, 0.24 nm, 1.15 nm, 1.87 nm, 2.31 nm	Higher attachment of diatoms was observed on rougher coatings
Sousa *et al.*, 2009 [38]	8	*Staphylococcus epidermidis* (9142, 9142-M10, PIA-positive 1457, 1457-M10, PIA-negative 1457, IE186, IE214, IE75) (bacteria)	2	acrylic silicone	0.79 nm 4.24 nm	Attachment of bacteria is higher on rougher silicone, however it cannot be treated as a pattern due to nanoscale of roughness
de Souza and Ferragut, 2012 [56]	Mixed culture	periphyton (algae)	1	polyethylene	5.08 µm, 10.19 µm 15.61 µm	Roughness of materials did not affect attachment
Preedy *et al.*, 2014 [164]	2	*Staphylococci, Streptococci* (bacteria)	1	BSA coating (bovine serum albumin) on glass	1.54 nm, 52.62 nm 81.87 nm, 103.63 nm 145.88 nm	Surface roughness affects the extent of adhesion forces
Sekar *et al.*, 2004 [31]	1	*Nitzschia amphibia* (bacteria)	2	titanium, stainless steel	grit-polished: 120, 220, 320, 400, 600, 800 grit	Rough materials had higher attachment than smooth materials
Singh *et al.*, 2011 [165]	2	*Escherichia coli, Staphylococcus aureus* (bacteria)	2	titanium film on glass, glass	16.2 nm, 21.7 nm 25.5 nm, 32.2 nm 5.12 nm	Attachment of bacteria increases with surface roughness, but only to some extent. For R_a>20nm it drops.
Sreekumari *et al.*, 2001 [54]	1	*Pseudomonas* sp. (bacteria)	1	stainless steel 304	3 µm	Bacteria attach more to rougher parts of stainless steel (welded area). Attachment decreases with increasing grain size
Morgan and Wilson, 2001 [57]	1	*Streptococcus oralis* (bacteria)	1	acrylic	0.43-6.44 µm (around 59 roughness values)	Attachment increased with surface roughness of cold-cure denatures acrylic plugs. Heat-cured acrylic did not show the same pattern
Köhler *et al.*, 1999 [55]	10	*Needle diatoms, Licmophora* sp.,*Vorticella* sp., *Zoothamnium commune, Suctoria, Hydrozoa, Mytilus edulis, Polydora* sp.,	1	glass	smooth, 100 µm 500 µm, 1000 µm 5000 µm	Microorganisms attached less on smooth surface.

		Balanus improovisus (bacteria, diatoms)				
Johnson, 1994 [39]	1	*Halosaccion glandiforme* (microalgae)	1	epoxy putty	smooth (100-1200 µm) nad rough (1-2 cm)	Attachment was higher on rough substrates
Hsu *et al.*, 2013 [166]	3	*Escherichia coli, Listeria innocua, Pseudomonas fluorescens* (bacteria)	2	alumina, silica	4.9 nm, 0.3 nm 6.7 nm, 6.4 nm 5.2 nm	Topography of materials has an influence on the attachment of bacteria- they form certain attachment pattern in relation to surface features.
Cui *et al.*, 2015 [35]	2	*Nannochloropsis oculata, Scenedesmus dimorphus* (microalgae)	6	nylon, glass, stainless steel	37.6 nm, 60.7 nm 124 nm, 57.8 nm 45.4 nm, 0.26 nm	Attachment of cells increases with surface roughness, when material of similar chemical composition is considered.
Bagherifard *et al.*, 2015 [169]	mixed culture	bacteria	1	stainless steel 316	0.09 µm, 5.92 µm 8.14 µm	Attachment of bacteria decreases with increase in surface roughness
Truong *et al.*, 2010 [170]	2	*Staphylococcus aureus, Pseudomonas aeruginosa* (bacteria)	1	titanium	0.22 µm, 0.08 µm	Both bacteria attached better to smoother equal channel angular pressing (ECAP) polished titanium
Gross *et al.*, 2016 [36]	1	*Chlorella vulgaris* (microalgae)	4	aluminium, stainless steel, polyester, polyethylene, nylon, polypropylene	smooth and mesh	Texture of square pore enhanced cell attachment

Table 5 Surface physical properties correlation with primary microbial adhesion

Reference	Number of species	Microorganisms types	Number of materials	Material types	Outcome
Ista *et al.*, 2004 [37]	2	*Ulva linza* (microalgae), *Cobetia marina* (bacteria)	6	SAM (self-assembled monolayer)	The attachment increases with decreasing water contact angle
Cui *et al.*, 2015 [35]	2	*Nannochloropsis oculata, Scenedesmus dimorphus* (microalgae)	3	nylon, glass, stainless steel	The adhesion of microalgae is strongly related to SFE of material. Attachment increases with decreasing SFE of substrate
Fletcher and Pringle, 1985 [174]	8	*Bacillus filicolonicus, Bacillus pacificus, Micrococcus* sp., *Flavobacterium uliginosum, Pseudomonas* sp, *Corynebacterium erythrogenes, Vibrio fisheri* (bacteria)	1	polystyrene	Attachment is decreasing with increasing SFE of surface
Sokolova *et al.*, 2012 [30]	3	*Cellulophaga lytica* (bacteria), *Navicula incerta* (microalgae), *Amphibalanus Amphitrite* (diatoms)	1	xerogel coatings	Adhesion strength is not related to SFE of coatings
Sousa *et al.*, 2009 [38]	8	*Staphylococcus epidermidis* (9142, 9142-M10, PIA-positive 1457, 1457-M10, PIA-negative 1457, IE186, IE214, IE75) (bacteria)	2	acrylic silicone	Adhesion capability is not related to surface hydrophobicity (Contact angle(CA)/SFE)
Klein *et al.*, 2014 [173]	1	*Navicula jeffreyi* (diatom)	5	stainless steel 316L, polytetrafluoroethylene, glass, polyamide-nylon, polyethylene	Hydrophobic substrates were preferred by diatoms
Gross *et al.*, 2016 [36]	1	*Chlorella vulgaris* (microalgae)	4	aluminium, stainless steel, polyester, polyethylene, nylon, polypropylene	Tetradecane contact angle had a significant correlation with cell attachment, although no relation was found for surface free energy, water or glycerol contact angle

Higher number of materials would need to be tested in order to determine the influence of SFE and surface hydrophobicity on the number of attached microalgal cells.

2.5 Application of attached systems

2.5.1 Microalgal biofilms in wastewater treatment

Limited research has been conducted on microalgal biofilms devoted to biofuels production [19]. Most of the studies are on application of algae biofilms to treat wastewater. Those methods of nutrient remediation have certain advantages. They operate at low temperature and pressure, and there is no requirement for catalyst [175]. In addition, biofilm processes are not only environmentally friendly treatments, but also effective in terms of operational costs [176] [177]. Examples of reactors to treat wastewater with the use of microalgal biofilms are as follows:

– PPMB Reactor [178]: The Parallel Plate Microalgae Biofilm Reactor (PPMB) was designed to immobilize nutrients from chemically treated household wastewater. Nitrogen and phosphorous were removed by the algal biofilm. The overall removal efficiencies of the system were satisfactory, 67% removal of total nitrogen and 96% removal of total phosphorous. The amount of total chemical oxygen demand and suspended solids was also reduced (by 74% and 82%, respectively).

– VSB Reactor [175]: The Vertical Submerged Biofilm Reactor (VSB) was used to remove nitrogen and sulfide from synthetic wastewater. The fixed-bed reactor made of polyvinyl chloride was able to remove 82.7% of total nitrogen and 98.2% of sulfide at the third stage of the process.

– EBT Reactor [177]: In this experiment, *Chlorella sorokiniana* was growing on walls of Enclosed Biofilm Tubular Reactor (EBT). In a reactor made from transparent polyvinyl chloride, a microalgal biofilm was used to treat piggery wastewater. Algae biofilm was capable of removing carbon, ammonium, and phosphate.

– MBB Reactor [179]: Apart from domestic wastewater, microalgae biofilms can be also used in treatment of raw water polluted by industrial activities. In 2013, Zhang tested Moving-Bed Biofilm Reactor (MBBR) for nitrogen removal, obtaining promising results.

– PRBC Reactor [180]: Microalgae biofilms are helpful in removing nitrogen and phosphorous, but they can be also used in lowering the concentrations of heavy metals, such as copper, nickel or manganese. Photo-Rotating Biological Contractor (PRBC) was used to attach algae and microbes, which were efficiently removing heavy metals from mining wastewater. From 20 to 50% of various heavy metals were taken away by the biofilm deposited on polyvinyl chloride disks partially immersed in acid mine drainage. Algae-microbial biofilm was able to withdrawn metals such as zinc, antimony, selenium, cobalt, aluminium and, as mentioned earlier, copper, nickel, and manganese. The summary of those studies is given in Table 6.

Table 6 Biofilm reactors to treat wastewater with removal efficiencies

Reactor	To clean	Reactor/culture volume	Removal efficiency [%]						
			TN	TP	COD	TSS	TOC	S^{2-}	NH_4-N
PPMB [178]	domestic wastewater	3L + 6L	67	96	74	82	-	-	-
VSB [175]	synthetic wastewater	18L	82.7	-	-	-	-	98.2	-
EBT [177]	swine slurry	7.5L + 0.5L	94-100	70-90	-	-	61	-	94
MBB [179]	raw water from Taihu Lake	45L	-	-	-	-	-	-	63.1
Attached algal culture system [181]	dairy from wastewater	0.05L + 0.15L	79	90	-	-	-	-	-

TN- total nitrogen
TP- total phosphorous
COD- chemical oxygen demand
TSS- total suspended solids
TOC- total organic carbon
S^{2-}- sulfide
NH_4-N- ammonium

2.5.2 Microalgae biofilm in biofuels production

There are only few studies on microalgal biofilms devoted to biofuel production [159]:

– Effect of nutrient starvation on lipid content: Nutrient starvation is so far the most common approach to increase fatty acids content in suspended cultivation

[159] [182]. Unfortunately, the same effect on microalgae growing in biofilms was not observed. Lipid content was not elevated by nutrient starvation for *Scenedesmus obliquus* and *Nitzschia palea* [19]. After three days of starvation, the concentration of lipids did not change and stayed on the level of 15% and 6% for *N. palea* and *S. obliquus*, respectively. When cultured in suspended mode, the same algal strains reached lipid level of 30% (*N. palea*) and 17% (*S. obliquus*) after three days of starvation. Nutrient starvation was not increasing the lipid content of microalgae, when grown in biofilms.

– Rotating Algal Biofilm Cultivation System: Gross constructed a Rotating Algal Biofilm cultivation system, in which he tested 16 materials as attachment surfaces [21]. The reactor was partially immersed in liquid medium. Rotations of reactor allowed the biomass that grows on substratum to alternatively enter liquid rich in nutrients and atmosphere with higher concentration of carbon dioxide. Similar approach was presented year earlier by Christenson and Sims (Figure 12) [32]. Their reactor achieved much better results regarding the biomass and fatty acid methyl esters (FAME) productivity in comparison with reactors in which microalgae were cultured in suspended mode [32]. Both studies showed that the best material for microalgae attachment is cotton. It is cheap, easy to acquire and as an attachment surface allows microalgae to achieve the highest biomass yields [21] [32].

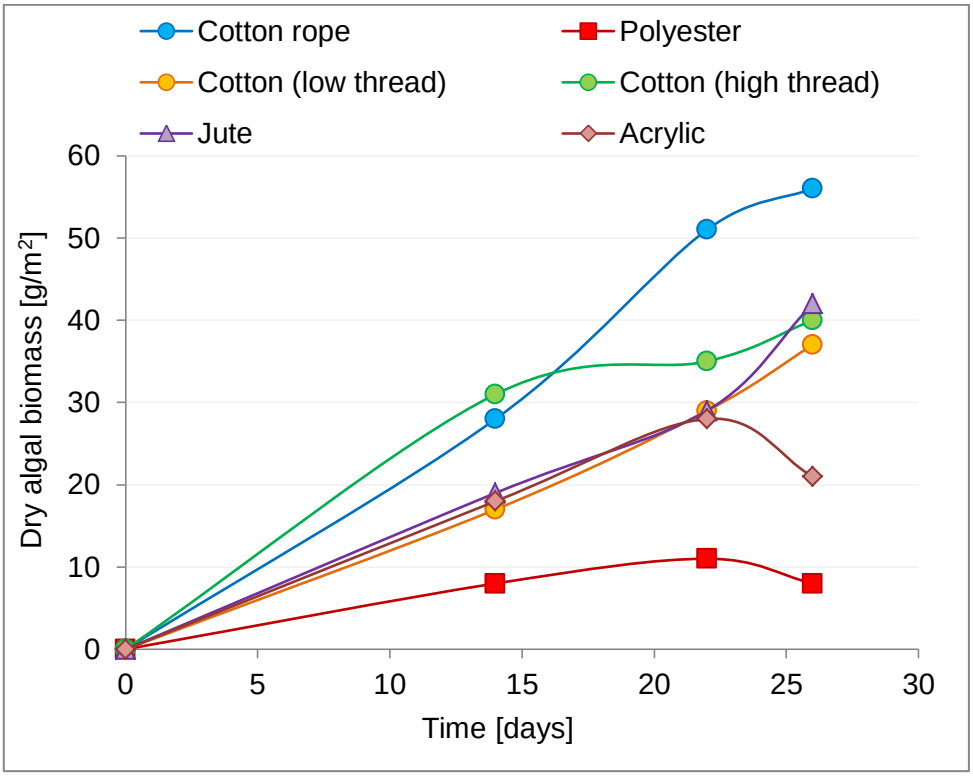


Figure 12 Growth of mixed microalgae culture on different materials [32].

– Attached Algal Culture System: In 2009, Johnson constructed a system intended to grow *Chlorella* species for biofuel production with simultaneous nitrogen and phosphorous removal [181]. Materials tested as a substrate were polystyrene foam, cardboard, polyethylene landscape fibre, loofah sponge, polyurethane foam, and nylon sponge. Among all these materials, the best in terms of biomass and total fatty acids production was polystyrene foam [181]. It was also easy to remove an algal biomass from this material and re-use polystyrene after the process (Figure 13). The material to attach cells was placed at the bottom of moving tank. Without water movement algae tended to accumulate at the bottom and created sediment rather than attach to the substratum. System was able to produce 3.2 g/m^2/day of microalgae. The lipid content was around 9%, which is much higher in comparison with maximal 5% of terrestrial crops. Dairy wastewater was applied as a medium. Removal of total nitrogen and total phosphorous reached level of 79% and 90%, respectively [181].

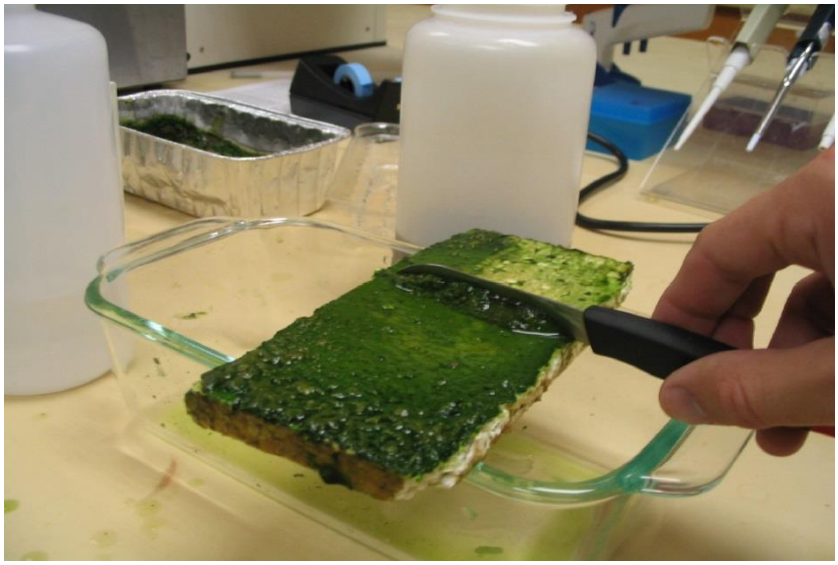


Figure 13 Removing microalgae biofilm from attached cultivation system [181]

2.5.3 Other experiments

Apart from systems to treat wastewater and produce microalgae for biofuels, there are other researches on microalgal biofilms applications:

– Light/Electricity Conversion System [183]: Biofilm can be applied to obtain energy. Biofilm-Based Light/Electricity Conversion System was developed to exchange light irradiation energy into electric current. Green algae were used in the experiment, however they were working only in the presence of heterotrophic bacteria. When the light reaches the reactor, extracellular electron transfer takes place. Electric current is generated.

– BOD removal: It is also possible to remove Biological Oxygen Demand (BOD) by application of microalgal biofilm inside Flat Plate Photobioreactor (FPP) and Tubular Packed Photobioreactor (TPP) [132]. Microalgal-bacterial biofilm is created either on beds carriers or strictly on reactor's walls. From both approaches, the second one is the most convenient, as it is not possible to achieve stability when biofilm is attached to bed carriers. In both biofilm reactors (FPP and TPP), removal rates of 92 and 108 mg BOD/L/h were achieved, in comparison with 77 mg BOD/L/h achieved in ordinary suspended reactor. It means that it is possible to conduct efficient BOD removal process with the use of microalgae and bacteria biofilm. However, the process still has certain

drawbacks. Photoinhibition lowers the operation performance and the biomass accumulation during growth phase could result in reactor blockage [132].

2.6 Aeroterrestrial microalgae: research needs and application in non-enclosure cultivation

There is a strong need for a research on aeroterrestrial microalgae which focus on their applications in industry. Present studies, as mentioned earlier, are directed towards biofouling prevention. The potential of aeroterrestrial microalgae in biofuel production, pharmaceutical industry and other areas is not known. It is essential to investigate their properties and applications, so they can be compared with aquatic species. In particular, growing aeroterrestrial microalgae by non-suspended cultivation is the most intuitive step in research that should be taken as soon as possible. Aeroterrestrial species naturally forming biofilm communities and highly resistant to environmental stresses are an attractive solution for emerging non-enclosure methods of cultivation. Their ability to draw moisture from humid air will be highly beneficial in systems where biofilm is cultivated outside liquid medium.

Some aquatic species may be highly sensitive to changes in environmental conditions, which leads to drop in products recovery. In contrast, resistance to desiccation and high tolerance to ultraviolet radiation of aeroterrestrial species can significantly improve the biofilm cultivation process.

This review shows that non-suspended cultivation is a promising approach in microalgae culturing, and from the natural tendency of aeroterrestrial microalgae to create biofilm it can be assumed that this kind of cultivation will be the most appropriate. Numerous advantages of aeroterrestrial species over aquatic microalgae, in particular at aerial settings, justify their selection for further work on the novel humid biofilm bioreactor that will be presented in following chapter of this thesis.

2.7 Non-enclosure methods for non-suspended microalgae cultivation: research needs

The review highlighted advantages of currently researched non-suspended microalgal cultivation systems. Higher biomass yields, lower energy and water consumption, cost effectiveness, efficient light distribution and easier maintenance of biofilm bioreactors are the main benefits of non-suspended systems over traditional, suspended cultivation methods. Non-enclosure approach of non-suspended cultivation in which cells are freely growing in biofilm communities is more effective way of microalgal biomass production than encapsulation methods. It is simpler, cheaper and does not involve any gelling agents.

Non-enclosure cultivation requires further research, in particular to aid optimisation and commercialisation of already existing biofilm systems. Investigation on novel concepts is also an important aspect. The review showed that aeroterrestrial microalgae could be potentially applied in aerial biofilm bioreactor, improving overall system performance. Therefore, the concept will be developed and investigated further in this thesis.

2.8 Conclusions

Growing microalgae in attached non-suspended systems is a novel concept. Biomass yield is comparable or higher than the same species grown at suspended mode of culturing. Consumption of water is much lower, which contributes to decrease in production costs. Distribution of light is improved, as it is not limited by culture density. Most of the cells are attached to substrate; only small part is free-floating within the medium and absorbing the light. The most significant advantage of attached systems is easier harvesting step. It was proven that the water content of microalgae scrapped from substratum is comparable to this of biomass after centrifugation. Avoiding this expensive and time-consuming step makes the algae production more feasible. Application of aeroterrestrial microalgae can potentially decrease the costs of production even more. The usage of water will be reduced to minimum, as algae will be grown in humid atmosphere, not in the medium. The light distribution is expected to be

enhanced, as no medium or floating cells would be absorbing the light. In addition, maintenance, mechanisation, and scaling-up of the whole system should be easier, as huge volume of water does not obstruct operations inside the reactor. Future work includes design of special reactor, in which humid atmosphere rich in CO_2 will be created and maintained. In addition, selection of proper substrate material is important, as there are no studies on the most effective substratum to grow aeroterrestrial microalgae. To find out whether this kind of microalgae could be feasible competitor to ordinary microalgal cultivation, those investigations should be carried out in the nearest future.

3 Impact of strains and materials' properties on primary attachment of aeroterrestrial and freshwater microalgae

3.1 Abstract

A broad study analysing primary adhesion of 36 substrate-strain pairings was conducted to address research gap on microalgal biofilm formation in relation to material and strain properties. Attachment of freshwater microalgae *Haematococcus pluvialis* and *Chlorella saccharophila* was compared to adhesion of aeroterrestrial microalgae *Stichococcus* sp. and *Chlorella luteoviridis*. The study suggested that there was no direct correlation between average substrate roughness or surface free energy (SFE) of materials and microalgal cell attachment. However, surface topography related to adhesion quality and quantity. Structural features of materials provided shelter from shear forces within liquid medium, although the access to nutrients inside niches might have been limited. There was no visible difference in attachment between aeroterrestrial and freshwater strains. Adhesion quantity was related to polarity of microalgal strains, presence of material features and elastic modulus of plastic materials.

3.2 Introduction

Primary colonisation is the fundamental step in microalgal biofilm development. The formation of a conditioning layer on the substrate followed by initial cell attachment, are the steps that control further biofilm development [104] [105]. Microalgae attach to surfaces forming different structures that help supporting cells' growth and biofilm development. Well-structured biofilm allows easier and more efficient transport of nutrients and can increase the metabolic activity of cells. During attachment process, most of the species capable of biofilm formation rely on extracellular polymeric substances (EPS) secretion. EPS producing cells formulate larger biofilms and accumulate more biomass in comparison to non-EPS producing strains [184]. In addition, their growth is more rapid. However, not all species mentioned in this study are able of EPS secretion. Some of them would be forced to exploit EPS producers for their own

benefit, weakening the biofilm [185]. For remaining non-producers, mechanism of attachment depends on attractive forces between the cell and substratum. An example of such strain is *Chlorella vulgaris*, not able to produce EPS on its own. It was shown that acid-base interactions play an important role in *C. vulgaris* adhesion [186].

Biofilm formation is affected by various factors such as: nutrients and water availability, light intensity [23] [24], chemical and physical properties of microalgae and attachment substrate [27] [28] [29], pH [31], irradiance [25], flow of medium and cell concentration in medium [26] [129]. Substrate and microalgal strain properties are other essential factors that significantly impact primary deposition of cells. The key finding from literature review enclosed in Chapter 2 is that the literature on substrate and strain properties and its impact on microalgal primary attachment is very limited. So far, most of the work on primary attachment had been done on bacteria and yeasts. Relatively little is known on microalgae attachment to substrates [187] and on the factors that influence microalgal biofilm formation. Some quantitative data is available, although no structure of algal primary colonisation has been analysed so far. Moreover, studies reported in the literature investigate a small number of strains and substrates. Most of the studies were performed on one or two-algae strains and only few materials in terms of their applicability as carrier substrate. Materials such as plastics, steel, glassand fibres were tested [30] [31] [36] [54] [39] [57] [164] [165] [170].

Impact of cell size on its propensity to adhere was not investigated: only the relation between cell size and the size of material structural features was analysed [143] [144]. Review on physico-chemical properties of microalgae revealed that hydrophobic cells prefer to adhere onto hydrophobic surfaces [149]. Some studies suggest that microbial attachment increases with increasing average surface roughness of the substrate [30] [31] [39], whereas other studies did not find such relation [56]. Attachment on hydrophobic materials is higher than hydrophilic substrates [37] [173], as hydrophobic interactions are involved at the first stage of biofilm formulation [82] [172]. Cui *et*

al. [35], Fletcher and Pringle [174] suggested that surface free energy (SFE) is another factor impacting adhesion to surfaces. According to their work, microalgae attachment increases with decreasing SFE of adhesion carrier. However, other studies showed that there is no correlation between SFE of material and microbial adhesion [30] [36] [38], Furthermore, there is a lack of studies on attachment patterns or formation factors for aeroterrestrial microalgae, which naturally grow in biofilms in terrestrial environments. The purpose of these researches in most cases, has been to determine an effective way to prevent biofilm formation or cell resistance to desiccation stress [13].

To address research gaps mentioned in paragraphs above, this chapter investigated aeroterrestrial structural attachment patterns and the influence of strains and materials properties, in particular the effect of average surface roughness and physico-chemical properties of materials on primary colonisation. The study considered a wide spectrum of materials and four strains of algae. Cellular properties of microalgae were also analysed and linked to their adhesion propensity: cell morphology expressed by equivalent cell diameter, circularity, roundness, cell area and volume; physico-chemical properties of microalgal cells and structure of attachment aggregates analysed by SEM imaging.

3.3 Materials and methods

3.3.1 Microalgal strains, materials and growth conditions prior to experiments

3.3.1.1 Microalgal strains

For primary attachment experiments, four microalgal strains from the same subphylum were selected: two aeroterrestrial species (*Chlorella luteoviridis* and *Stichococcus* sp.) and two freshwater species (*Chlorella saccharophila* and *Haematococcus pluvialis*). Selection included two different habitat-based types of microalgae to illustrate potential difference in attachment mechanisms between species naturally growing at aerial conditions and species that are found in water environment.

All selected microalgae are known to exhibit certain properties or consist of components that are of industrial interest, aiding potential commercial application of novel cultivation method proposed in this thesis. *Stichococcus* sp. is capable of remediating acid-extractable organics mixture isolated from oil sands [188]. Ruffell *et al.* [188] showed that this microalga is able to significantly reduce the AOE marker ion, indicating a promising solution for a new bioremediation strategy. *C. luteoviridis* was able to efficiently remove nutrients from municipal wastewater, so it can be applied in wastewater treatment plants [189]. *C. saccharophila* can be considered as an attractive candidate for biofuel production. Tan and Johns [190] showed that almost 50% of the cell weight was lipids, while Isleten-Hosoglu *et al.* [191] results indicated that lipid content in cell was equal up to 37% dry weight. Moreover, oleic and linoleic acids were the principal fatty acids in *C. saccharophila*. Oleic acid can constitute up to 80% of total fatty acid methyl esters in *C. saccharophila* [192]. High concentrations of oleic acid are highly desirable in biodiesel industry. *H. pluvialis* is a commonly known source of beta-carotene, astaxanthin [2]. Astaxanthin is widely used as a pigment in aquaculture, mainly applied as a fish feed for salmons, lobsters and shrimps [193]. *H. pluvialis* is able to accumulate the highest amounts of natural astaxanthin in comparison to other organic sources [194]. Zhang *et al.* [195] tested *H. pluvialis* in biofilm bioreactor, reaching astaxanthin productivity up to 160 mg/m^2/day. It is very good result when compared to other researches [76] [3] [196] [197]. The system applied two stages simultaneously; astaxanthin-rich red cells were localised at the top of biofilm layer, whereas green cells were localised at the bottom [195].

Haematococcus pluvialis (CCAP 34/7), *Chlorella luteoviridis* (CCAP 211/3), *Stichococcus* sp. (CCAP 379/30) and *Chlorella saccharophila* (CCAP 211/57) were obtained from the Culture Collection of Algae and Protozoa (CCAP), UK. Prior to the experiments, all strains were grown in Bold Basal medium inside an Innova®44 incubator shaker at 180 RPM under continuous light illumination of 3110 lux and temperature of 22°C.

3.3.1.2 Materials

For primary attachment purposes, nine different materials were tested: stainless steel 304 bead blasted (304 BB; ACO Technologies® plc, UK), stainless steel 304 sand polished (304 SP; ACO Technologies® plc, UK), stainless steel 316 bead blasted (316 BB; ACO Technologies® plc, UK), stainless steel 316 sand polished (316 SP; ACO Technologies® plc, UK), polyvinyl chloride (PVC), polyethylene terephthalate (PETG; Bay Plastics Ltd, UK), Swiss float glass (glass; VWR®), polystyrene (PS; FisherBrand, UK) and polypropylene (PP; Pondskipper Crafts; UK). Materials were chosen to include material types mostly researched at biofilm settings: plastics, metals and glass. Plastic and steel materials were proved to be efficient attachment substrates [31] [33]. The selection was made based on physico-chemical and topographical properties of materials. Materials cover wide spectrum of surface roughness, ranging from 0.13 μm to 6.69 μm. Each material type has smooth and rough equivalents, to better illustrate eventual differences in microalgal attachment. Most of chosen materials are hydrophobic, in order to promote cell deposition. Glass was selected as a reference material, to illustrate how well cells can attach to the hydrophilic substrate which lacks textural features.

3.3.2 Microalgae morphology analysis

ImageJ software was used to evaluate the morphological properties of microalgae: cell size (minor, major and equivalent diameter), cell area, cell perimeter, cell circularity and roundness.

Cell area was calculated by ImageJ software based on 2D selection outlining exact area of inspected cells. The volume of cells was calculated manually. A particle can be treated as a sphere when its circularity exceeds 0.78 [198], although the shape of microalgal cells observed in this study was not ideally spherical. All cells resembled the shape of spheroid; therefore the volume of cells was calculated with the use of major (D_{max}) and minor (D_{min}) diameters for oblate and prolate spheroids.

3.3.3 Surface texture analysis

Average surface roughness and roughness factor was measured by an Olympus Lext OLS3100 confocal microscope. 50x lenses were used to ensure high precision of results. the tiling technique allowed to measure surface samples of approximately 1mm^2, increasing the accuracy of the measurement.

3.3.4 Surface free energy measurement of substrates

The measurement of contact angle of all substrates was conducted with the use of a Biolin Scientific Theta Lite Optical Tensiometer equipped with OneAttension Software, using sessile drop technique. Water, diiodomethane and ethylene glycol were used as reference liquids of known surface free energy (water γ_l=72.80 mN/m; diiodomethane γ_l=50.80 mN/m; ethylene glycol γ_l=48.00 mN/m) [199].

Prior to measurements, materials were cleaned with propanol. Water/diiodomethane/ethylene glycol droplet was dropped onto the dried material surface. The OneAttension software analysed the contact angle.

The contact angles of rough substrates were corrected with the use of Wenzel roughness factor [200]. The roughness factor is a ratio between actual surface area and geometric surface area (Equation 1).

$$W_{rf} = \frac{actual\ surface}{geometric\ surface}$$

Equation 1 Wenzel roughness factor

The relation between measured contact angle and actual contact angle of rough substrate can be expressed by the formula below (Equation 2):

$$cos\theta_m = W_{rf} \cdot cos\theta$$

Equation 2 Measured contact angle

Where:

θ_m- measured contact angle

θ- actual contact angle

W_{rf}– Wenzel roughness factor

Interfacial free energy between substratum and reference liquids was evaluated with the OneAttension software based on contact angle measured by optical tensiometer. Calculations were conducted based on the Owens and Wendt [201], Rabel [202] and Kaelble [203] (OWRK) and Fowkes [204] method. Geometric mean approach was used in order to obtain the value of dispersive and polar components of surface free energy. When combined with Young's equation, it became Equation 3:

$$\boldsymbol{\gamma_l(1+\cos\theta) = 2 \cdot [\sqrt{(\gamma_l^p \gamma_s^p)} + \sqrt{(\gamma_l^d \gamma_s^d)}]}$$

Equation 3 Geometric approach

where:

ɣ- surface free energy

d- dispersive component of surface free energy

p- polar component of surface free energy

l- liquid

s- substrate

After rearrangement, according to Owens and Wendt [205]:

$$\boldsymbol{\frac{\gamma_l(1+\cos\theta)}{\sqrt{\gamma_l^d}} = \sqrt{\gamma_s^p} \cdot \frac{\sqrt{\gamma_l^p}}{\sqrt{\gamma_l^d}} + \sqrt{\gamma_s^d}}$$

Equation 4 OneAttension geometric approach

The Attension software plotted two graphs against each other: $\sqrt{\gamma_l^p}$ / $\sqrt{\gamma_l^d}$ and $\gamma_l(1+\cos\theta)$ / $\sqrt{(1+\gamma_l^d)}$. It found the $\sqrt{\gamma_s^p}$ value as a slope, and $\sqrt{\gamma_s^d}$ value as y-intercept. Total free surface energy is a sum of its polar and dispersive components.

3.3.5 Surface free energy measurement of medium and microalgae

Liquid tension of Bold Basal medium [206] was measured by Du Noüy Ring method [207] with the use of a Krüss K6 ring tensiometer.

Surface free energy of microalgae was measured by the sessile drop technique proposed by Busscher *et al.* [208]. A Biolin Scientific optical tensiometer was used to evaluate contact angle. Prior to measurements, each strain was cleaned three times with distilled water and filtered through Whatman® cellulose acetate membrane filter with 0.45 µm pore size. Even distribution of cells within the microalgae layer was confirmed by confocal microscope analysis. The water contact angle was recorded every five minutes in order to evaluate the time required by the microalgae to dry. Drying time was strongly dependent upon the strain. A drop of water/diiodomethane/ethylene glycol was dropped onto dried microalgal layer. The shape and size of the drop forming on the substrate layer was analysed in the same manner as for material substrates.

3.3.6 Primary attachment

3.3.6.1 Inoculation

The culture was pre- grown to end of lag phase, to a final concentration of $4.7\pm0.5\cdot10^{6}$ cells/ml. Carrier substrates were immersed in a pre-grown microalgal culture inside a polypropylene container and kept in darkness for around 16 hours at temperature of 22°C. This particular time of contact between attachment substrate and microalgal culture was selected to obtain an optimum between colonisation rates on material and further cell multiplication in liquid medium. Shorter times resulted in too small inoculum, whereas longer time of contact caused increased growth of cells in liquid phase. Higher number of cells in liquid medium corresponded to higher number of cells that could adhere. It would disturb the reading of actual number of adhered cells, for example indicating that more cells adhered than there was available at the beginning of inoculation.

Inoculum was evaluated by Equation 5:

$$\rho_i = \frac{V \cdot \rho_{cell}}{A_{container}}$$

Equation 5 Inoculum

where

ρ_i- inoculum [number of cells/cm^2 of substrate]

ρ_{cell}- cell density of the culture [number of cells/mL of microalgal culture]

V- volume of the microalgal culture poured into container [mL]

$A_{container}$- area of container bottom [cm^2]

3.3.6.2 Primary attachment measurement

Biofilms inoculated on materials were removed from the container. Prior to sonication, materials were mildly washed by a continuous stream of distilled water to remove loosely attached microalgal cells. The surfaces were immersed in a known amount of phosphate buffer solution (pH=7.4, 10ml) in a closed container placed in the Fisher Scientific FB 15051 ultrasonic bath at 37 kHz frequency. To remove firmly attached cells, substrate with attached microalgae was sonicated for 5 minutes for 1-6 times until complete removal. Complete removal of cells was investigated optically under Olympus CX41 microscope.

The number of cells in the buffer solution after sonication gave the total amount of firmly attached cells per sample. Cell density was measured by cell counting method under an Olympus CX41 microscope with the use of Improved Neubauer Haemocytometer (Weber England, depth 0.1mm, 1/400 mm^2) together with optical density method carried out by a Jenway 6800 Double Beam UV/ViS Spectrophotometer. Attachment was given in the number of attached cells per cm^2 of material (Equation 6).

$$\rho = \frac{\rho_{buff} \cdot V_{buff}}{A_{sample}}$$

Equation 6 Primary attachment

where:

ρ- primary attachment [number of cells/cm^2 of substrate]

ρ_{buff}- cell density of the buffer solution [number of cells/ml of buffer]

V_{buff}- volume of the buffer [mL]

A_{sample}- area of sample material [cm^2]

3.3.6.3 Scanning Electron Microscope Analysis

Stichococcus sp. attachment was investigated with Field Emission Guns Scanning Electron Microscope Nova NanoSEM 630 (FEG SEM) to evaluate attachment pattern on different substrates. Attachment was observed by secondary electrons (SE) signal. Images were modified with the use of Gimp 2.8.10 GNU Image Manipulation Program, to mark microalgal cells embedded in EPS.

Confocal images of substrates were taken by Olympus Lext confocal microscope. Images were transformed into 3D images with the use of the Olympus Lext software, to illustrate characteristic structural features of materials. Image in Figure 22 was transformed into 3D with open source community software for quantitative bioimage analysis Icy 1.6.1.1.

3.4 Results and discussion

3.4.1 Primary attachment

The number of attached cells was measured after overnight inoculation, the results are presented in Figure 14.

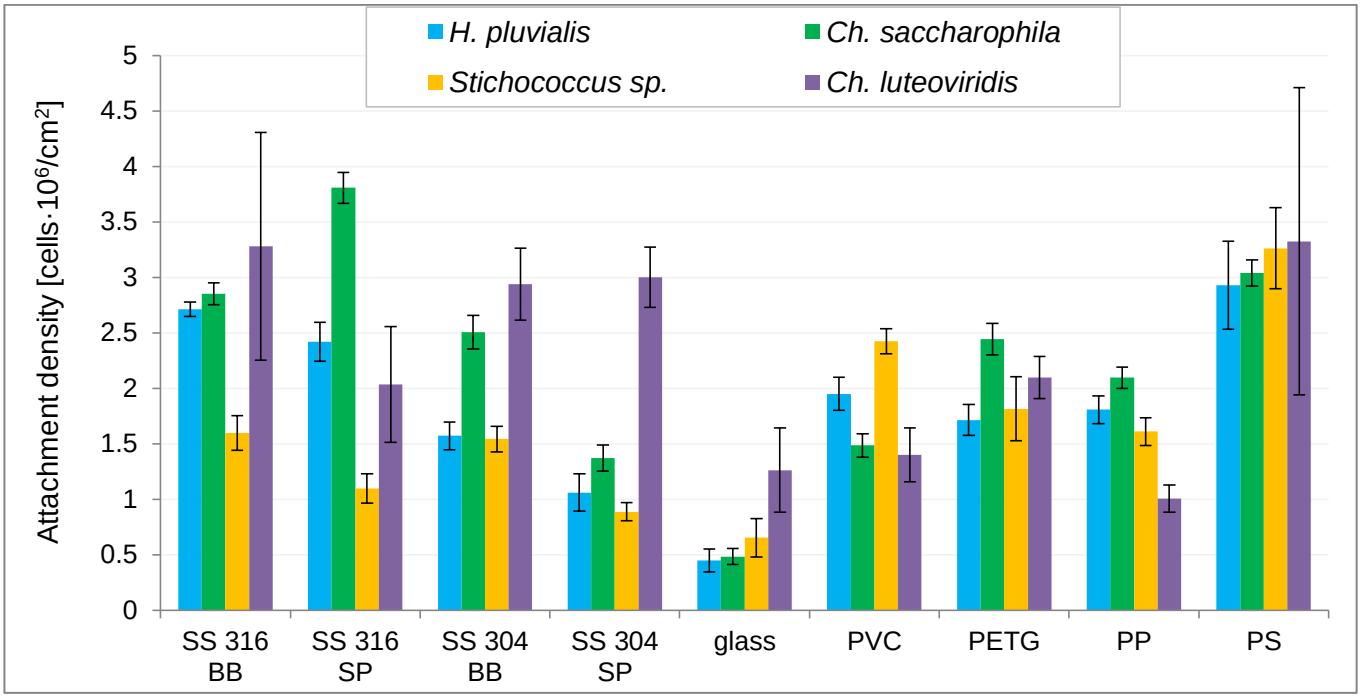


Figure 14 Primary attachment of microalgae strains

The lowest level of attachment was observed for glass. All four microalgal strains did not attach well to this material. The lowest level of attachment were achieved by *H. pluvialis* with only $4.5 \cdot 10^5$ cells attached per cm^2 of glass.

The highest level of attachment was on PS, all microalgae tested adhered well to PS, with 2.9±0.4, 3.0±0.1, 3.3±0.4 and 3.3±1.4 cells·$10^6/cm^2$ for *H. pluvialis, C. saccharophila, Stichococcus* sp. and *C. luteoviridis*, respectively.

In general, all strains attached more on bead blasted steel than on sand polished, 2.4±0.6 cells·$10^6/cm^2$ attached to bead blasted steel in comparison to 2.0±0.9 cells·$10^6/cm^2$ that attached to sand polished steel. *Stichococcus* sp. attached in higher numbers to 304 and 316 BB (1.6±0.03 cells·$10^6/cm^2$) in comparison to 316 and 304 SP (1.0±0.1 cells·$10^6/cm^2$).

An efficient inoculation would allow all the cells available in the inoculum to adhere onto the surface. In this experiment between 9% and 81% of the inoculum attached to the surfaces depending on microalgal strain and material type. The average colonisations of all strains did not exceed 50%, which was not a very positive result. Material-strain pairing needs to indicate high inoculum

colonisation, preferably close to 100%. This is an important aspect in aeroterrestrial/liquid-free methods of microalgae cultivation [50]. Cells attached during inoculation would be the only cells available for biofilm growth, as no co-aggregation can take place in non-liquid reactors. There are no free-floating cells present in mist/humidity, therefore additional cell settlement is not possible. The idea of non-liquid cultivation relies on high initial colonisation, corresponding to a more effective utilisation of the inoculum.

The comparison between freshwater and aeroterrestrial microalgae indicated that both types of microalgae can successfully attach to substrates. It was also observed that the characteristics of attachment are dependent upon material and genus rather than isolation habitat. Both species from *Chlorella* genus, *C. luteoviridis* and *C. saccharophila* showed better attachment than *H. pluvialis* and *Stichococcus* sp. on most materials. Although *C. luteoviridis* had high attachment it was characterised by high error, implying high variability. Low variability in primary adhesion among the same sample is crucial when assessing process viability in industrial applications.

3.4.2 Primary attachment profile of aeroterrestrial *Stichococcus* sp. microalgae

Image analysis was performed in order to unveil complex aeroterrestrial microalgae attachment patterns and explain why they occur. Project limitations allowed investigating primary adhesion by SEM of only one strain among the ones tested in this thesis. Biofilm patterns of aeroterrestrial species via SEM imaging were not investigated before, therefore this section focuses on *Stichococcus* sp.. Moreover, among all tested strains, *Stichococcus* sp. secreted the highest amount of EPS, making it easier to observe EPS presence in freshly established biofilm. Primary attachment of *Stichococcus* sp. was investigated with secondary electrons (SE) signal. All pictures obtained were in greyscale. Microalgae cells embedded in EPS, were marked with green colour (Figure 15 to Figure 20).

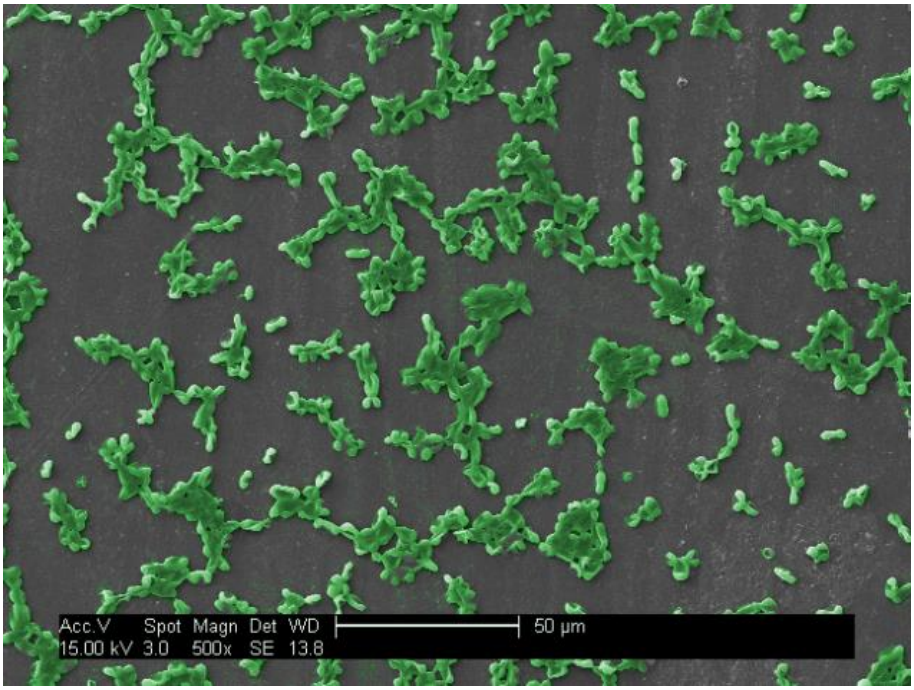


Figure 15 SE SEM image of *Stichococcus* sp. on PETG; x500 magnification

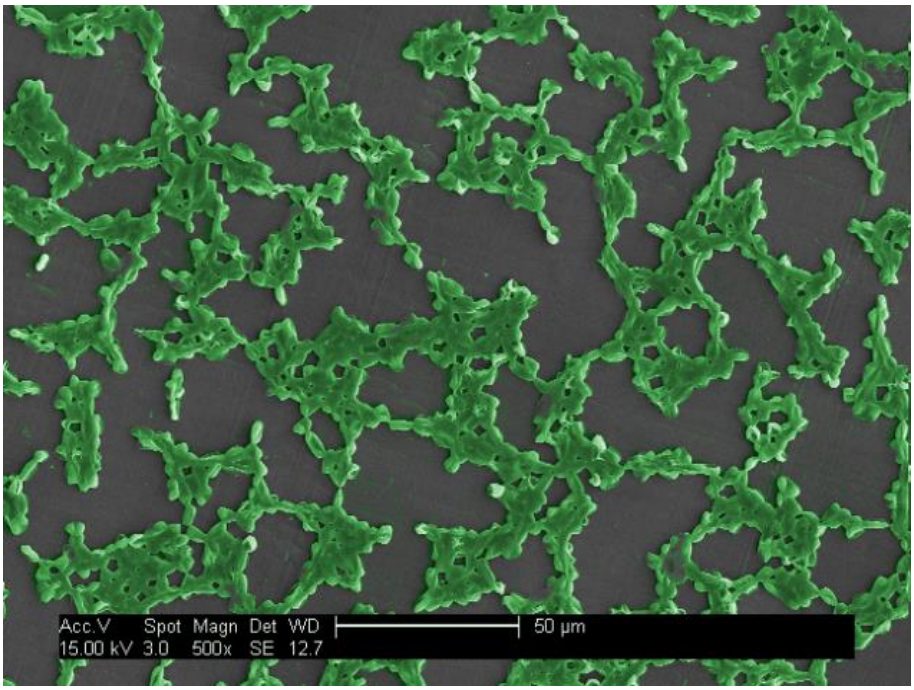


Figure 16 SE SEM image of *Stichococcus* sp. on PP; x500 magnification

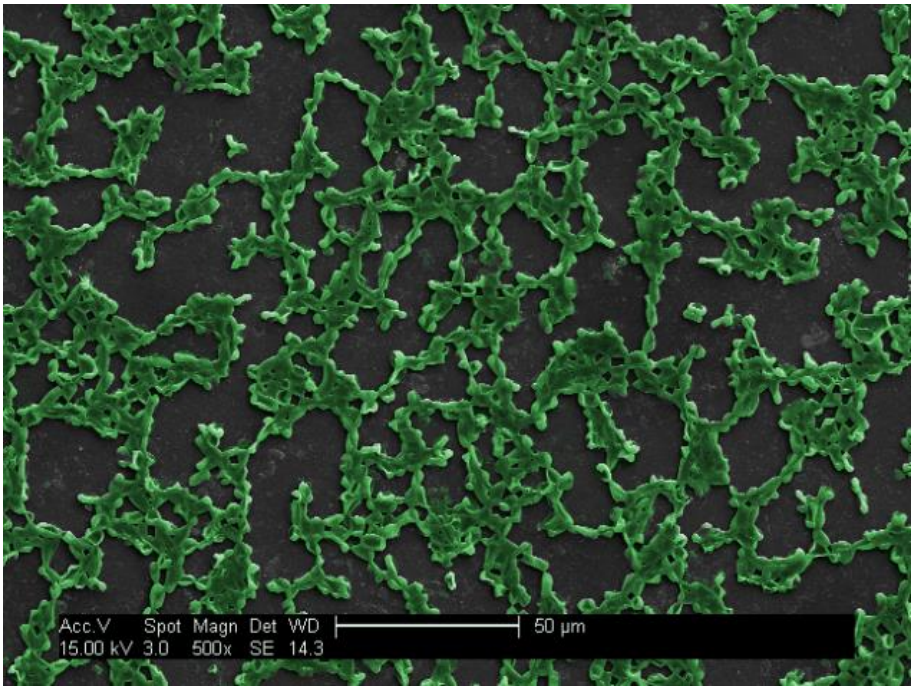


Figure 17 SE SEM image of *Stichococcus* sp. on PVC; x500 magnification

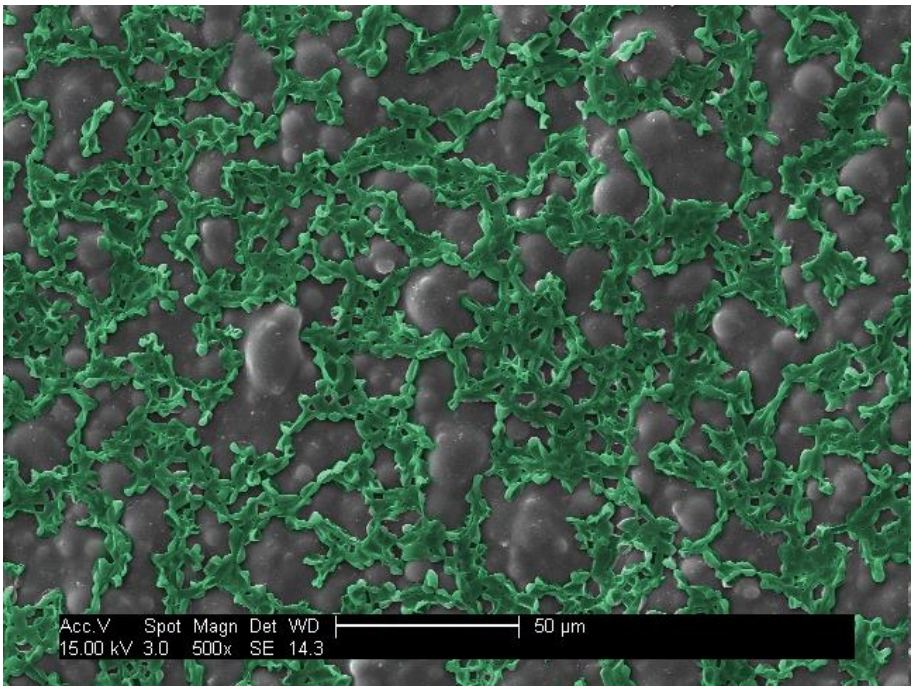


Figure 18 SE SEM image of *Stichococcus* sp. on PS; x500 magnification

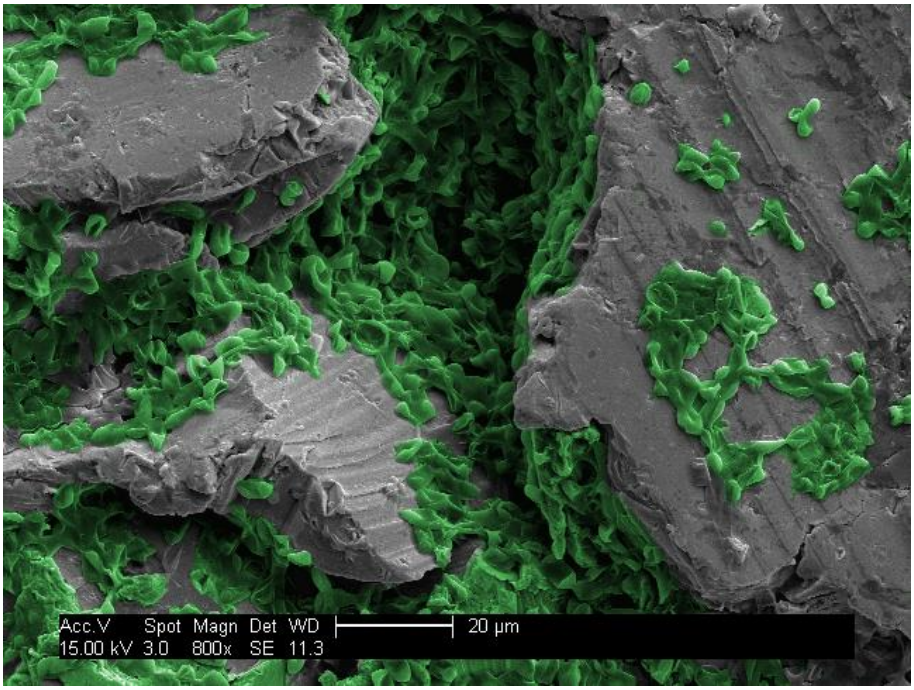


Figure 19 SE SEM image of *Stichococcus* sp. on bead blasted steel (x800 magnification)

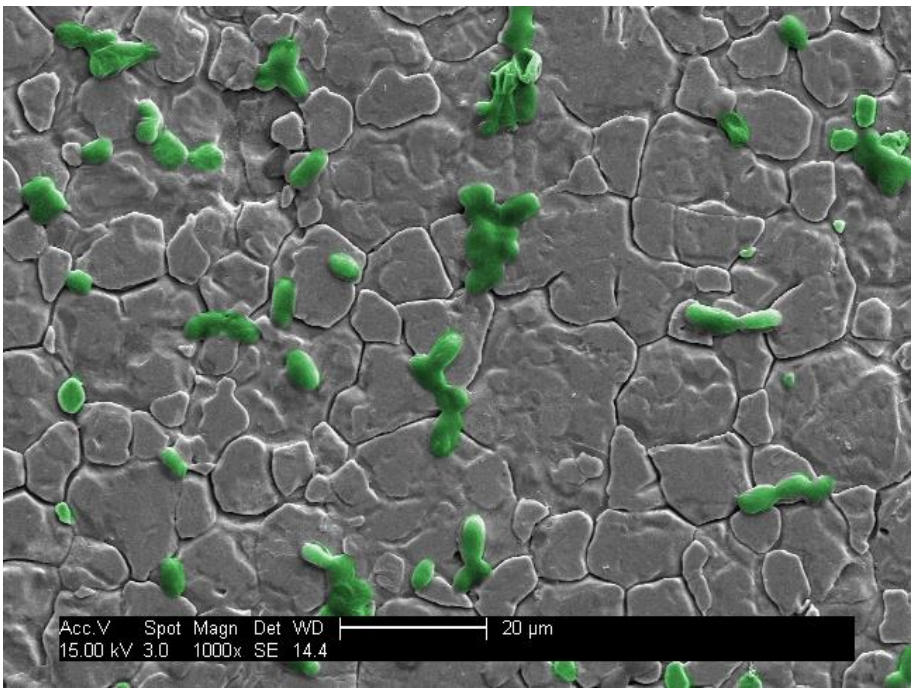


Figure 20 SE SEM image of *Stichococcus* sp. on sand polished steel (x1000 magnification)

SEM image analysis showed significant differences in structure amongst the tested materials. There was a visible difference in surface roughness between

bead and sand polished steel of the same composition (0.29 versus 6.69). The roughest substrate was bead blasted steel (Figure 19), PETG was the smoothest plastic (Figure 15), while PS had 'bubble like' features (Figure 18). Significantly higher number of cells attached to bead blasted steel, because they accumulated inside the cavities (Figure 19). Two times more *Stichococcus* sp. cells settled on SS 316 BB than on SS 304 SP, which was clearly visible on the SEM images (1.6 against 0.9 cells·10^6/cm^2). Denser aggregates on PVC and PS in comparison to PP and PETG corresponded to the higher number of cells attached (2.4 cells·10^6/cm^2 and 3.3 cells·10^6/cm^2 compared to 1.6 cells·10^6/cm^2 and 1.8 cells·10^6/cm^2, respectively).

A well-organised pattern of attachment is visible in particular on plastic materials. The adhesion behaviour of *Stichococcus* sp. on PETG, PP, PVC and PS was very similar to the aggregation pattern observed by Sousa *et al* [38] for *Staphylococcus epidermidis* bacterium on silicone (with a surface roughness equal to 0.789 nm). These researchers suggested that the adhesion pattern was a consequence of the unique features of the substrate

The same conclusion can be drawn from the results in this chapter. In the case of 316 BB steel, *Stichococcus* sp. microalgae were forced to form aggregates around specific surface features, and were not able to arrange themselves in structured chains (Figure 19). Whereas, on smooth surfaces, microalgae were freely allowed to settled everywhere and form optimised interlinked colonies (Figure 15- Figure 17).

3.4.2.1 Gravitational forces influence attachment pattern

All the inoculation steps were done on plates laying horizontally in a liquid solution. Gravitational forces have an influence on the adhesion pattern [209]. When biofilm is formed on vertically arranged substrates, its metabolic activity can be reduced [210]. For example, in the work of Cavalcanti *et al* [210] only the horizontally arranged disks of polymethylmethacrylate (PMMA) resulted in cell settlements with characteristic patterns. When PMMA disks were placed vertically *Candida albicans* cells were weakened by gravity and did not produce well-structured biofilms.

Figure 21 shows *Stichococcus* sp. attachment on bead blasted steel after horizontal inoculation. Most of the cells gathered inside the surface cavities, whilst others distributed along specific features of the substrate. This is especially visible at the bottom of substrate's elevations, where cells accumulated. Moreover, due to shear and gravitational forces, cells stack on each other forming dense clusters. The presence of cavities can enhance primary attachment, as more cells can be entrapped inside them. Entrapped cells can be more resistant to water flow, although excessive accumulation can limit access to nutrients.

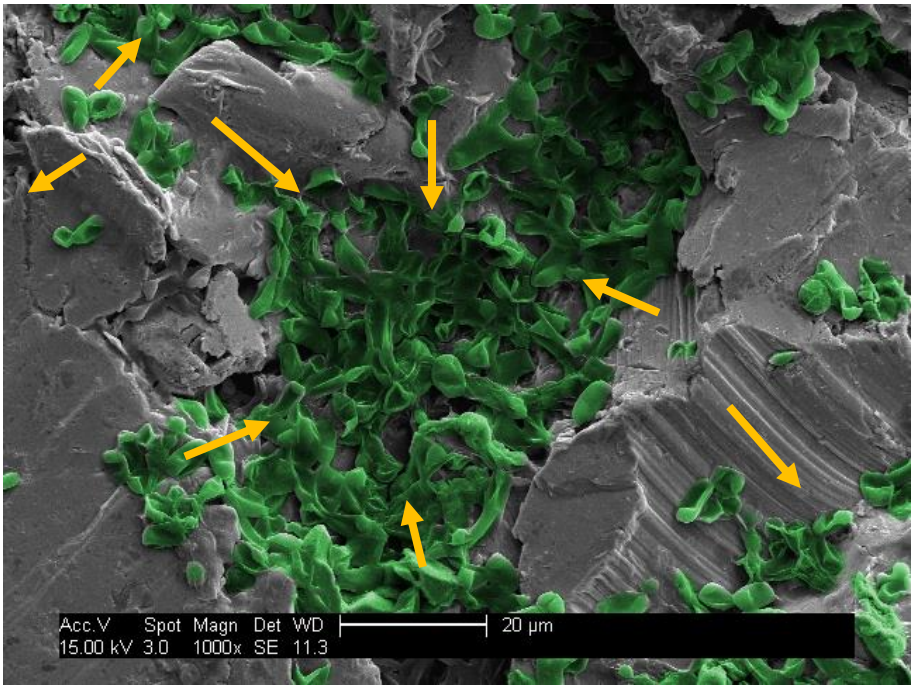


Figure 21 SE SEM images of *Stichococcus* sp. on 316 BB steel; x1000 magnification. Gravitational forces indicated by arrows (→)

Stichococcus sp. attachment to a nylon mesh is a good example of gravity influenced adhesion.. A nylon mesh was one of the materials tested in preliminary experiments on microbial adhesion. Even though nylon mesh was not an effective attachment substrate and was not chosen for further primary attachment experiments, *Stichococcus* sp. exhibited an interesting adhesion pattern on this material. Primary adhesion on nylon net is showed in Figure 22. Similar mechanism of attachment as in the case of *C. albicans* was observed on

this material. *Stichococcus* sp. cells were trying to overcome gravitational forces; therefore formation of well organised aggregates was not possible. Cells were shifted from the top of a nylon wire towards its edges, which corresponded to distribution of gravitational forces.

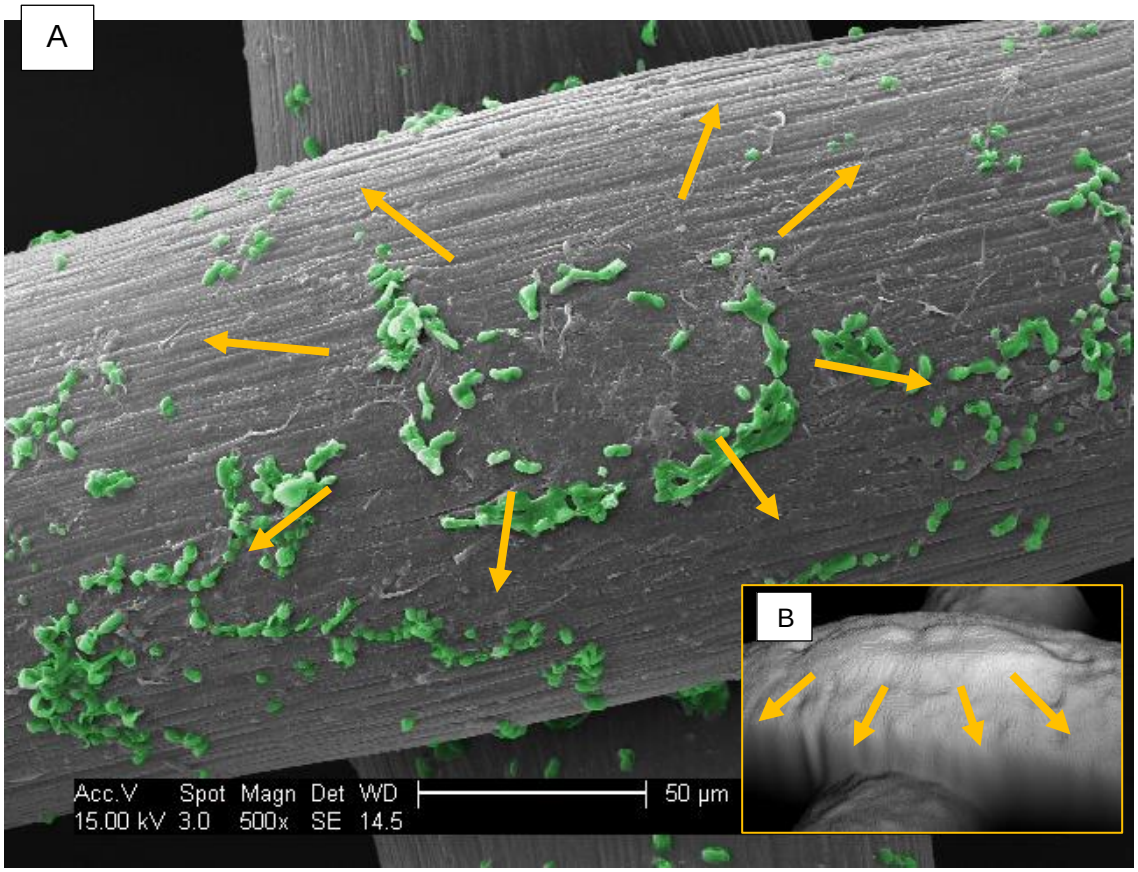


Figure 22 SE SEM images of *Stichococcus* sp. on nylon net; x500 magnification (A) and 3D image of nylon net; x100 magnification (B). Gravitational forces indicated by arrows (→)

No attachment pattern was formed on both 316 bead blasted steel and nylon mesh. It was not possible for *Stichococcus* sp. cells to overcome gravitational forces. As mentioned before, more uniform arrangement of cells improves metabolic efficiency; therefore it is very important to inoculate samples horizontally.

3.4.2.2 pH influences attachment pattern

pH of surrounding medium is an important factor that can impact on the formation of well-structured microcolonies. Hamadi *et al* [211] showed that the

adhesion behaviour of *Staphylococcus aureus* bacteria on glass was affected by the pH of the medium. Structured biofilm patterns were observed at neutral and alkaline pH (6.5 and 9) rather than at strongly acidic or strongly alkaline pH (5 and 11) [211].

The pH of the BB medium inoculum in this chapter was 7.6±0.5, which is close to the pH optimum reported for *Stichococcus* sp. (pH 8.2) [212] [213]. Therefore, microbial attachment took place in conditions favourable to *Stichococcus* sp. and all the materials in this chapter were inoculated at conditions favourable to the formation of well-organised biofilm structure.

3.4.3 Microalgae morphology analysis

The circularities of *H. pluvialis, C. luteoviridis, Stichoococcus* sp. and *C.saccharophila* are 0.89, 0.81, 0.82 and 0.88, respectively (Table 7). Cell diameters of microalgae vary from 5.19 µm for *C.luteoviridis* to 6.24 µm for *Stichococcus* sp. However, given the error, it was assumed that all the equivalent diameters of tested microalgal strains are the same. The difference in volume was also assumed not to be significant. From the cell size distribution analysis it could be also concluded that cell division and growth were taking place simultaneously and all cultures were at exponential growth phase at the same time [214]. In lag phase no cell division takes place, cells grow in size only, which results in more uniform distribution of cells [215]. In general, the morphological properties of all strains were very similar.

Table 7 Morphological properties of microalgae

	H. pluvialis	*C. luteoviridis*	*Stichococcus* sp.	*C. saccharophila*
Major diameter [µm] D_{max}	6.24±1.42	7.57±1.11	8.12±1.37	6.13±1.20
Minor diameter [µm] D_{min}	5.40±1.33	4.25±0.80	4.37±0.56	5.06±1.01
Equivalent spherical diameter [µm] D	5.84±1.37	5.19±0.78	6.24±0.86	5.60±1.08
Cell area [µm^2] A	26.41±13.49	31.34±8.38	34.05±9.64	24.05±9.02
Cell perimeter [µm]	18.76±4.46	21.90±3.05	22.74±3.25	18.15±3.61
Cell volume [µm^3] Oblate spheroid $V = \frac{\pi}{6} \cdot D_{max}^2 \cdot D_{min}$	105.61±32.76	120.98±27.50	147.68±22.27	108.48±35.66
Cell volume [µm^3] Prolate spheroid $V = \frac{\pi}{6} \cdot D_{min}^2 \cdot D_{max}$	90.95±28.79	69.50±22.70	80.82±15.63	90.44±32.19
Circularity	0.89±0.04	0.81±0.07	0.82±0.05	0.88±0.03
Roundness	0.91±0.04	0.60±0.10	0.58±0.07	0.88±0.06

3.4.4 Microalgae morphology relation to primary attachment

The total number of cells attached onto the different substrates was related to the equivalent diameter of the cell. For some materials, a relation between the size of the cell and primary attachment of microalgae was found. However, given that the cell size are very similar for all the species analysed, a broader range of cell sizes would need to be tested, in order to draw conclusions on the relationship between cell size and primary attachment. Moreover, this relationship could be also dependent upon the roughness of the substrate.

Other morphological characteristics such as circularity, roundness, cell area or cell perimeter were also not related to primary attachment. When microalgal predispositions to create biofilm are considered, it can be presumed that cell morphology is less important than its physico-chemical properties.

3.4.5 Surface texture analysis

Surface roughness varied from 0.13 µm for glass up to 6.69 µm for bead blasted steel (Table 8). Based on the material type, the substrates could be separated as plastic and stainless steel materials. Each type of material has a smoother and a rougher representative. 316 SP and 304 SP sand polished steels, had a flawless mirror-like finish and low surface roughness (0.29µm and 0.43µm, respectively). In contrast, 316 BB and 304 BB steel surfaces had low reflectivity with a uniform, mat structure of very high average surface roughness, 15 to 20 times higher than sand polished steel.

Table 8 Morphological properties of materials

Material	Average surface roughness, R_a [µm]	Wenzel roughness factor, W_{rf}	Geometric surface area [mm^2]	Actual surface area [mm^2]
Glass	0.13±0.01	1.01±0.00	0.80±0.00	0.81±0.00
SS 316 SP	0.29±0.01	1.03±0.00	0.80±0.00	0.83±0.00
SS 304 SP	0.43±0.01	1.20±0.00	0.80±0.00	0.96±0.01
SS 304 BB	6.46±0.27	2.06±0.07	0.80±0.00	1.65±0.06
SS 316 BB	6.69±0.29	2.15±0.06	0.80±0.00	1.72±0.05
PETG	0.22±0.04	1.02±0.01	0.80±0.00	0.82±0.01
PP	0.31±0.02	1.01±0.00	0.80±0.00	0.81±0.00
PVC	0.66±0.19	1.05±0.03	0.80±0.00	0.84±0.02
PS	1.51±0.05	1.18±0.04	0.80±0.00	0.95±0.03

Polystyrene (PS) was the roughest among the tested plastics. Its average surface roughness was 1.51 µm - two times higher than PVC (0.66±0.19 µm).

The difference between smooth PETG and PP roughness was not that significant (0.22μm and 0.31μm, respectively). PETG and PP were moderately rigid materials, while PVC and PS possessed high elasticity and bended easily.

Glass was tested as an additional material, because of its significantly different roughness and physico-chemical characteristics. Surface roughness of glass was the lowest among all materials tested (0.13μm), which was reflected in its structure. The material did not possess any textural features; therefore the attachment of microalgae was difficult and based on cell-to-substrate interactions mostly.

3.4.6 Surface roughness relation to primary attachment

The results from this study showed that there was no evident correlation between surface roughness and primary attachment of cells. The attachment was not significantly higher on bead blasted steels, which had an actual available area two times bigger than the remaining substrates. In addition, experiments on plastics, steel and glass materials showed that microtopography does not play an essential role in attachment for cells with size around 6-7μm. Similar conclusions were made by Sousa *et al.* [38], who concluded that the material's nanotopography cannot be considered as an attachment factor for bacteria. It was showed that surface roughness not always affects deposition [56] [57]. Presence of features, however, affected the aggregation pattern of microalgal cells (biofilm quality), which was visible on SEM images of *Stichococcus* sp., as shown in Figure 18. The same conclusion was made in smaller study conducted by Scheuerman *et al.* [171], who tested *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* bacterial strains adhesion to silicon.

3.4.7 Physical properties of strains and substrates

. Dispersive and polar components of surface free energies were given for each algae specie and material (Table 9).

Table 9 Physical properties of microalgae and materials

Microalgae strain/Substrate	Contact angle [°]			Surface free energy ɣ [mJ/m^2]		
	Water	Diiodomethane	Ethylene glycol	γ^{tot}	γ^{d}	γ^{p}
H. pluvialis	90.26±3.17	44.80±2.00	33.27±4.21	39.589	37.428	2.161
C. luteoviridis	96.44±7.51	52.69±5.61	66.79±0.74	32.875	32.277	0.598
***Stichococcus* sp.**	23.59±1.93	62.71±6.99	75.27±6.38	48.69	18.666	30.024
C. saccharophila	58.65±6.33	58.01±2.30	72.50±2.74	38.1	36.885	1.215
SS 316 SP	59.54±4.23	36.97±0.82	40.42±2.80	48.321	37.976	10.345
SS 304 SP	63.00±4.98	40.27±0.81	75.50±1.99	39.263	32.234	7.030
SS 304 BB	59.07±2.16	55.95±3.06	66.21±4.07	38.749	23.704	12.854
SS 316 BB	70.64±8.63	58.73±3.61	83.14±4.23	30.604	23.704	6.900
PETG	85.02±2.55	35.61±4.71	59.79±1.25	41.092	39.734	1.358
PP	58.09±11.13	62.10±3.83	64.79±4.80	35.991	23.606	12.385
PVC	103.64±2.67	61.71±0.98	61.53±7.30	29.755	28.407	1.348
PS	88.03±1.65	37.67±1.50	66.74±1.15	39.167	38.36	0.807
Glass	23.58±2.00	35.21±4.62	46.15±6.15	58.112	34.192	23.92
Bold Basal Medium	N/A	N/A	N/A	71±0.4	21.3±0.1	49.7±0.3

The dispersive and polar components of the surface free energies of microalgae can be compared to the study performed by Ozkan and Berberoglu [217]. They tested 7 different green algae strains. Dispersive components (γ_c^d) ranged from 9.7 to 37.8 mJ/m^2, while polar components (γ_c^p) ranged from 0.0 to 5.1 mJ/m^2. The data obtained in this chapter was very similar, with the exception of the polar component of *Stichococcus* sp. (γ_c^p=30.024 mJ/m^2). However, this value might still be appropriate as some diatoms possess higher polarities [217].

All microalgal strains had lower polarity than water. Therefore, with respect to the thermodynamic equation for adhesion, the attachment should take place on

surfaces possessing lower polar surface energy and higher disperse surface energy (Table 9) [218]. Therefore, it was determined that the attachment would take place on all materials.

Contact angles of 316 BB, 304 BB, 304 SP and PS were corrected with Wenzel factor [200]. The remaining materials due to negligible roughness factor were not corrected. Glass was the only hydrophilic material, with a water contact angle of 23.58°. The remaining substrates were either slightly or strongly hydrophobic, which were favourable for the promotion of biofilm formation [155]. The SFE of glass was within the 43.10-112.90 mJ/m^2 range given by Krüss [219]. The SFEs of steel and plastic materials were also comparable to measurements performed by other scientists [219].

Bold Basal Medium consists mainly of water with trace amount of nutrients. It can be assumed that surface free energy components of the liquid are the same as for water [220].

3.4.8 Physico-chemical properties of strains and substrates in relation to primary attachment

3.4.8.1 Physical properties of microalgae in relation to their primary attachment

Microalgae attachment capability was linked to their polarities, and decreased with its increase. *C. luteoviridis,* which possessed the lowest polarity, was the microalgae with the higher attachment values. This relation could be explained with the thermodynamic model. If all substrates have higher dispersive component than that of the medium ($\gamma_s^d > \gamma_l^d$) and lower polar component than that of the medium ($\gamma_s^p < \gamma_l^p$), then the strain with higher dispersive but lower polar energy would attach more. It was, however; still not clear why this phenomenon happened [33]. A possible explanation is that the cell surfaces are very complex and vary among each other, from both chemical and physical point of views. Polar component of surface free energy corresponds to Coulomb interactions that describe the magnitude of electrostatic force between two charged elements [221]. In this case, polar component described the repulsive forces between negatively charged microalgal cell and tested substrate.

Charges of materials and microalgal cells tested in this chapter are presented in Appendix D.2. More polar cells had to overcome higher repulsive forces when they were approaching the surface of attachment substrate.

Very poor adhesion propensity of *Stichococcus* sp. can be explained by its high polarity, reaching 30 mJ/m^2. Cells of *Stichococcus* sp. encounter higher cell-to-substratum repulsion, hence less cells would attach to the substrates. In the same time, *C. saccharophila* and *C. luteoviridis* had much lower polar part of SFE. Therefore, they had lower repulsive forces to overcome and their attachment was favoured.

All microalgal strains possessed very similar values of surface free energy, on average equal to 39.5±4.6 mJ/m^2. Consequently, microalgae possessing the lowest polar component of SFE compared to the remaining strains, would have the highest dispersive component of SFE. Dispersive part of SFE describes the temporary changes in charge distribution and is referred to van der Waals forces that are the main attractive forces acting on the cell that approaches carrier substrate [222]. Microalgae with dispersive component higher than polar component are the ones that are able to overcome repulsive forces and are able to attach to the substratum. The relation between dispersive and polar component was more important than the value of surface free energy itself. In this study, all strains had high dispersive component of SFE; although only *Stichococcus* sp. had a higher polar component. In theory, it would mean that these algae should not be able to adhere onto any substrate. However, in practice for some cells it was possible to successfully attach onto the different substrates. It was caused by the fact that, as mentioned in Chapter 3.2.2, cells are capable of adjusting to the environmental factors and change hydrophilic cells into hydrophobic [223]. Hydrophobicity of cell is strictly connected to the polar component of SFE; water contact angle was linearly related to the polarity of strains in this study. Therefore, the dispersive/polar components can be used to describe the propensity of microalgal cell to attach. As long as some van der Waals interactions are present, the attachment will occur, although not for the whole inoculum.

3.4.8.2 Physical properties of materials in relation to microalgal attachment

There was no visible relation between the surface free energy of materials and the primary attachment, which was also showed in Souza *et al* work [38]. Polar and dispersive components of materials' SFE were also not correlated to microalgal colonisation. However, primary attachment was connected to the substrates' hydrophobicity, determined by water contact angle. It was observed that microalgae tend to attach more onto hydrophobic steel and plastic materials than strongly hydrophilic glass, as also showed by Cooksey and Wigglesworth-Cooksey [155] and Ista *et al.* [37]. This preference was caused by hydrophobic interactions involved at the biofilm formation stage [82] [172]. Hydrophobic materials are good attachment substrates for hydrophobic microalgae, while hydrophilic cells attach more to hydrophilic materials [224]. This may explain why hydrophilic *Stichococcus* sp. attached well to glass (6.5±1.7 cells·10^5/cm^2). However, to confirm this statement, more hydrophilic materials would need to be tested and compared to hydrophobic materials.

3.4.8.3 The influence of steel materials on primary attachment of microalgae

304 and 316 grades stainless steels are nickel-chromium steels with a small content of carbon, manganese, silica, phosphor, nitrogen and sulphur. In addition, grade 316 contains molybdenum. Molybdenum is known for its biofilm development inhibition properties according to Percival [225]. However, in this study the adhesion to grade 316 was not worse than the adhesion to grade 304. It needs to be pointed out, that the study of Percival focused on pure molybdenum, not stainless steel containing molybdenum as one of the alloying elements [225]. In another study Percival *et al* [226] showed that microalgae tend to attach more to 304 steel (no molybdenum content) than to 316. However, significant differences on surface roughness between those two steel grades also influenced the results.

3.4.8.4 The influence of plastic materials composition on primary attachment of microalgae

Plastic materials are polymers build of synthetic organic compounds that consist of linear carbon atoms arranged in chains (PS, PP, PVC) or carbon chains containing other components such as nitrogen, oxygen or sulphur (PETG). Even though steel and plastic materials vary in chemical composition and structure, the attachment on both types of materials was comparable. Similar to steel materials, plastics are in general good attachment substrates. The highest attachment on the PS plastic was within the range 2.9-3.3 cells·10^6/cm^2, while the highest attachment on 316 BB steel was equal from 2.9 to 3.3 cells·10^6/cm^2.

All strains tested attached in the highest number to polystyrene material, on average, 3.1±0.2 cells·10^6 adhered to cm^2 of PS. Low error indicates that the attachment was almost the same for all microalgae. Polypropylene was the worst plastic for microbial adhesion; only 1.0±0.1 to 2.1±0.1 cells·10^6/cm^2 attached to this material. However, there was no significant difference in chemical composition between PS and PP. Both PS and PP materials have very similar properties: density (1.0 and 0.9 g/cm^3), dielectric strength (18-20 and 21-22 kV/mm) and strength-to-weight (32-44 and 32-37 kN-m/kg) [227]. The only difference was in impact strength and elastic modulus.

PS is characterised by higher elastic modulus than PP; 1.9-2.9 GPa compared to 0.9 GPa, respectively. Aldred and Clare [228] showed that low elastic modulus contribute to inhibition of cyprids settlement. The same pattern was observed in other work of Aldred *et al.* [229]. Attachment strength on polycarbonate was higher than on polydimethylsiloxane [229]. In this study, polypropylene had the lowest elastic modulus among tested plastics; PVC and PETG were characterised by higher ratio of stress to strain (1.4 and 2.2 GPa, respectively).

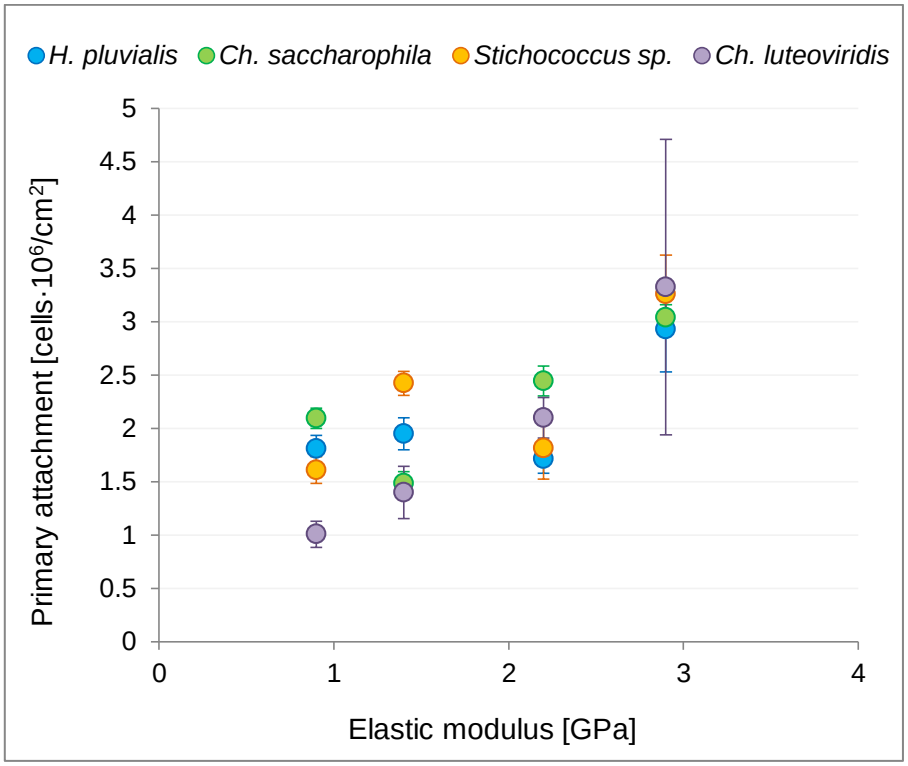


Figure 23 Elastic modulus of plastics in relation to primary attachment of microalgae

Therefore, due to the lowest elastic modulus, the attachment on PP substrate was the lowest in the relation to remaining plastics. In fact, the correlation between elastic modulus of the substrate and adhesion was clearly visible for all plastics-microalgae pairings, as shown in Figure 23. Correlation coefficients between primary attachment and elastic modulus of plastics were high: 0.73, 0.81, 0.73 and 0.98 for *H. pluvialis, C. saccharophila, Stichococcus* sp. and *C. luteoviridis*, respectively. However, elastic modulus did not have a direct impact on adhesion quantity. It rather had an influence on detachment rate of reversibly attached cells than directly influence the bio-adhesion. Fracture mechanics describes the importance of the elastic modulus of coatings and underlines its contribution to the cell detachment [230] [231]. It means that a number of already adhered cells would detach from less elastic material, which would contribute to the decrease in overall primary colonisation.

3.4.8.5 The influence of the glass material composition on primary attachment of microalgae

Microscopic slides used as a glass substrate in this study are made of float glass [232]. It is a material primarily consisting of silicon dioxide (75%), sodium oxide (13%) and calcium oxide (10%) and trace amounts of other oxides (aluminium, potassium, magnesium, iron (III), titanium and sulphur).

Glass was the least effective attachment carrier among all tested materials. As mentioned in previous chapters, it was mainly caused by its high polarity and very smooth surface that promoted higher shear forces of moving liquid. However, its chemical composition might have also had an influence on poor attachment. Akesso *et al.* [233] showed that SiO_2 coatings can be very effective inhibitors of attachment of green alga *Ulva* sp. and bacterium *Pseudomonas fluorescens*. Bioactive glasses containing similar oxides (SiO_2-Na_2O-CaO-P_2O_5) as glass tested in this study were proven to have antimicrobial properties [234]. They release ionic alkaline species that have disinfectant properties [235]. *Stichococcus* sp., *H. pluvialis* and *C. saccharophila* attached in the lowest numbers to glass: 6.5±1.7, 4.5±1.0 and 4.8±0.7 cells·10^5/cm^2. The only exception was *C. luteoviridis* which attachment was the worst on PP with 1.0±0.1 cells·10^6/cm^2, although the glass was second worst material for adhesion of this strain with 1.3±0.4 cells·10^6/cm^2. Therefore, apart from physical properties of glass tested, its chemical composition also affected the adhesion of all microalgal strains

3.5 Conclusions

In this study the factors influencing primary attachment of 4 algae species to 9 different materials were investigated, including the isolation habitat (aeroterrestrial vs freshwater), surface roughness (smooth vs rough materials) and physico-chemical properties of materials and algae (hydrophobicity vs hydrophilicity, polarity vs dispersivity). Primary attachment of freshwater microalgae was similar to the adhesion of aeroterrestrial species, naturally growing in biofilms. This was caused by the fact that both freshwater and aeroterrestrial microalgae are determined to survive in hostile conditions. To

prove whether aeroterrestrial or freshwater microalgae are more appropriate for biofilm cultivation, a proper investigation on their further growth needs to be carried out. This will determine whether freshwater species can reproduce on substrates at the same rate as aeroterrestrial ones, preferably in both liquid and terrestrial environments.

Microalga *Stichococcus* sp. was forming well-structured biofilm community on plastic materials. However, the presence of surface structural features affected the uniformity of attachment. The effect of gravity was observed on steel materials characterised by highly diverse structure; cells were accumulating in niches and did not form patterned aggregates. Accumulation resulted in high attachment on bead blasted steel, although the adhesion on smooth plastics was also very good. Smooth plastics promoted formation of well-structured aggregates.

The polarity of microalgae was an important factor when considering their adhesion ability. The primary attachment decreased with microalgal polarity, as cells had to overcome higher repulsive forces resulting from electrostatic interactions. There was, however, no relation between the polarity of materials and number of cell attached.

In this study, bigger cell size corresponded to lower attachment on steel materials; however the error in sizes was too high to draw meaningful conclusions. Microalgae with more uniform cell size distribution would need to be tested.

Material texture had significant influence on attachment pattern and biofilm quality, although there was no correlation between surface roughness and quantitative adhesion. Presence of adhesion favouring cavities that decreased the shear forces of flowing medium was more important than the value of average surface roughness. Anti-fouling molybdenum in 316 grade steel did not contribute to inhibition of biofilm deposition. Adhesion on plastics was related to their elastic modulus, as it influenced cell release from substrate and contributed to reduction of primary colonisation. Moreover, chemical composition of glass could inhibit the adhesion to this substratum.

The study indicates the importance of physico-chemical properties of materials and microalgae. Experiments on 36 pairings showed that attachment predictions are not straightforward and depend on many factors. Even though a lot of factors influencing attachment were kept constant, the characteristics of substrates and strains still had a significant influence on the attachment pattern and numbers.

4 Impact of material properties on freshwater and aeroterrestrial biofilm development in aerial conditions

4.1 Abstract

Chlorella luteoviridis, Chlorella saccharophila, Haematococcus pluvialis and *Stichococcus* sp. were grown on steel and plastic materials in aerial conditions to investigate the impact of physical and topographical properties of carrier substrates on biofilm development. The primary adhesion was promoted on rougher substrates, as it was shown by other studies. However, further biofilm development was better on smooth materials. Lack of structural features had a significant impact on biofilm formation quality, which contributed to increased nutrient transportation and cell multiplication. Hydrophobicity of materials influenced the development of *C. saccharophila* and *C. luteoviridis* biofilms, although no such correlation was found for *H. pluvialis* and *Stichococcus* sp.

4.2 Introduction

The switch from single-cell mode of growth into a biofilm-based community is highly advantageous for microorganisms as it provides shelter from hostile environmental conditions such as strongly acidic/alkaline pH, shear forces of moving liquid or antibiotics [236]. Biofilm formation and development are complicated processes and mechanisms governing microbial behaviour in biofilm are still not fully understood [23] [27] [51] [52] [53] [101]. Biofilms are ubiquitous in both aquatic [23] and terrestrial [9] [87] [88] environments. Hence, understanding biofilm characteristics is very important, as microbial communities play a crucial role in many biological and industrial processes. However, although beneficial to microorganism and microbial-based processes, biofilms can also cause significant problems in industry, causing bio-corrosion [10], decolourisation and weathering [9] of the substrates they colonise. As the majority of these processes take place in aquatic environment, most of the researches on microbial communities focus on these biofilms, whereas studies on terrestrial biofilms are very limited.

Biofilms in non-aquatic environments are the communities that colonise natural terrestrial environments and artificial substrates. They mainly consist of microalgae, bacteria, protozoa, fungi and cyanobacteria [9] [89]. The occurrence of aerial biofilms is mainly influenced by environmental conditions such as relative humidity (RH) level, temperature or nutrients availability [237]. That is why most studies on aerial biofouling mainly focus on environmental factors [80, 237] rather than on material substrate properties. In addition, the majority of studies on physico-chemical and topographical properties of materials focus on primary attachment of cells, further biofilm development is rarely discussed nor analysed. This is because usually biofilm formation is a detrimental phenomenon, and most studies focus on inhibition of biofilm formation rather than its growth. Materials which prevent attachment of microbial cells are desirable in many industries. However, very little is known on the carrier materials supporting the biofilm development in relation to their properties.

For example, hydrophobicity of materials has an influence on the primary attachment of microorganisms. Bacteria have proven to be able to adhere to both hydrophilic and hydrophobic materials [238], although cells prefer to adhere to hydrophobic materials, as hydrophobic interactions govern the attachment [82]. Araujo *et al.* [239] tested two materials as biofilm substrates for anaerobic granular sludge: glass, as hydrophilic substrate, and polypropylene (PP), as hydrophobic substrate. Similarly to the results presented in Chapter 3, initial attachment of *Archea* was promoted on the hydrophobic substrate; 460 cells·10^4/cm^2 adhered on PP in comparison to only 82 cells·10^4/cm^2 on hydrophilic glass. Yet, further biofilm development was very similar on both materials with 6.3 and 7.2 cells·10^4/cm^2 on glass and polypropylene substrate, respectively. Moreover, the growth rates were higher on glass than on PP: 0.54 compared to 0.34 day^{-1}. The data related to the growth was however limited and the authors could not draw any conclusions on the impact of materials' polarity on biofilm growth. A similar outcome was published by Lorite *et al.* [53] using *Xylella fastidiosa*; biofilm development was not influenced by substrate's hydrophobicity (glass or silicon). However, as the authors highlighted, the

number of materials tested was too small and no data was available on the surface structure of the materials. Differences in biofilm growth could not only be linked to surface polarity but also to surface topography.

In contrast, Gomes *et al.* [240], reported that on the four biomedical materials tested (glass, stainless steel, polyvinyl chloride and silicone) *E. coli* adhered in higher numbers to hydrophobic materials. Moreover, biofilm growth and development was also related to materials' hydrophobicity, with final biofilms density equal to 9.29±0.63, 14.2±2.08, 20.2±2.89 and 27.1±4.68 cells·10^7/cm^2 on glass, stainless steel, polyvinyl chloride and silicone, respectively. The corresponding water contact angles were equal to 47.0±0.4°, 67.0±1.7°, 79.3±0.9° and 115.4±0.4°, confirming that the hydrophobicity of the substrate had an influence on final biofilm density.

Study on primary attachment of cells to surfaces showed that microbial cells adhesion can be facilitated by the presence of attachment points [127]. In addition, structured surface can give cells shelter from shear forces of moving liquid [171]. The preference of cells attachment to structured substrates was also observed for algae in Chapter 3. However, little is known about the further multiplication of cells on structured materials. Gladis and Schumann [123] suggested that rougher materials may accumulate more moisture than smoother ones and favour the growth of phototrophic biofilms on roof tiles at aerial conditions. However, no other studies are available to confirm that statement. Chung *et al.* [12] showed that *Staphylococcus aureus* growth was significantly inhibited on structured Sharklet AF™ polydimethylsiloxane elastomer (PDMSe), when smooth PDMSe was used as a reference material. After 14 days of cultivation *S. aureus* covered around 54% of the total area of smooth PDMSe but only 7% of rough PDMSe. The structure of Sharklet AF™ was specially designed to inhibit *S. aureus* biofilm formation and development, with appropriate shape and size of rectangular ribs array. However, the primary colonisation of these bacteria was also significantly inhibited on this substrate, making it difficult to discern the two impacts. In another study, Leonhard *et al.* [241] showed no visible difference between *Candida albicans* and

Streptococcus salivarius cellular growth on smooth and rough medical silicone. The surface coverage after 19 days cultivation varied from 4% to 12% for both rough and smooth carrier substrates.

To the best of author's knowledge, there are no studies on microalgal biofilm growth at aerial conditions in relation to substrate properties; the majority of research focused on environmental factors and their influence on multispecies biofilms. Moreover, most of the studies investigated bacteria biofilms and tested limited number of strains and materials, focusing on only one aspect of material properties (surface roughness or hydrophobicity).

It was suggested that appropriate changes in structural characteristics of materials may contribute to biocide-free inhibition of biofilm development [123]. Therefore, this study focused on the impact of surface topography, hydrophobicity and polarity of materials on biofilm development of four microalgal species. Hydrophobic materials were chosen in order to promote initial colonisation of aeroterrestrial and freshwater microalgal strains. To minimise the influence of surface hydrophobicity on cellular adhesion, materials of similar water contact angle but various topography were analysed. The growth of biofilms was conducted in humid atmosphere, to mimic natural environment for aeroterrestrial microalgal growth.

4.3 Materials and methods

4.3.1 Microalgal strains and materials

Chlorella saccharophila (CCAP 211/57)*, Chlorella luteoviridis* (CCAP 211/3)*, Haematococcus pluvialis* (CCAP 34/7) and *Stichococcus* sp. *(CCAP 379/30)* were obtained from Culture Collection of Algae and Protozoa (CCAP), UK.

Sand polished (SP) and bead blasted (BB) stainless steel of grade 316 and 304, (ACO Technologies® plc, UK), polystyrene (PS) (FisherBrand, UK) and polyethylene terephthalate glycol modified (PETG) (Bay Plastics Ltd, UK) were used as carrier substrates.

4.3.2 Inoculation and growth conditions

Prior to experiments, microalgae were grown on Bold Basal Medium (BBM) in 250 ml Erlenmeyer flasks using an Innova®44 photobioreactor at 22°C, 3110 lux light intensity and constant 180 RPM. Microalgae were grown at continuous illumination as it stimulates the biomass and products yields in the case of *H. pluvialis* [242] and *Chlorella* species [243]. Stainless steels and plastics were treated with isopropanol prior to inoculation.

Inoculation of the substrates to support biofilm growth was performed with microalgal culture of known initial density (4.7±0.5 cells·10^6/cm^2) to allow biofilm formation. Materials were incubated overnight at 22°C in darkness following the method reported in [244].

Microalgal biofilms were grown horizontally in humid atmosphere maintained at 75-95% RH using a mini-fogger mist generator (Maplin, UK). The biofilms were maintained at 22°C and illuminated by fluorescent lamps at 3110 lux (42 µmol/m^2/s) light intensity at 12/12 light cycle. Unlike pre-growth, main experiments were conducted at 12:12 photoperiod, as intensive mixing is not applied when microalgae grow in biofilm [73]. Lack of mixing could lead to a potential oxidative stress in biofilm [245] and in a consequence decrease the biomass yields.

4.3.3 Biofilm density and growth rates calculations

Biofilm development was calculated by counting the number of cells attached to the substrates. Three sacrificial samples of each material-strain pairing were removed from the reactor at day 1,3 and 5. Substrates with biofilm were placed inside a beaker filled with a known amount of phosphate buffer solution (10 ml, ph= 7.4) and sonicated at 37 kHz frequency with a Fisher Scientific FB 15051 ultrasonic bath until the biofilm was removed (by checking removal progress every 10-30 seconds). The buffer solutions containing the suspended cells were counted using an Olympus CX41 optical microscope and a Weber England haemocytometer (Improved Neubauer, Depth 0.1mm 1/400mm^2) [246] . The number of cells/ml of buffer (ρ_{buff}) was converted to the total amount of cells in

the biofilm using Equation 7. In order to obtain biofilm density expressed in number of cells per unit area (ρ), the total number of cells in the biofilm was divided by the material surface area, A_{sample}.

$$\boldsymbol{\rho = \frac{\rho_{buff} \cdot V_{buff}}{A_{sample}}}$$

Equation 7 Number of cells in biofilm (biofilm density)

where

ρ - biofilm density

ρ_{buff} - cell density in buffer

V_{buff} - volume of buffer solution

A_{sample} - surface area of the sample

Growth rates of developing biofilm were calculated according to Wood [247]. Growth rate (r) describes the change in population [$\ln(\rho \cdot \rho_0^{-1})$] over time ($t-t_0$) and can be expressed by Equation 8.

$$\boldsymbol{r = \frac{\ln(\frac{\rho}{\rho_0})}{t - t_0}}$$

Equation 8 Biofilm growth rate

where

r- growth rate [day^{-1}]

ρ - biofilm density at t [$cells/cm^2$]

ρ_0- biofilm density at t_0 [$cells/cm^2$]

t- time of growth [days]

t_0- initial time of growth [days]

4.3.4 Physico-chemical properties of materials

The properties of the materials used in these experiments were discussed in Chapter 3. Water contact angle, surface roughness and polarity of materials are summarised in Table 10.

Table 10 Physico-chemical properties of the supporting materials

Material	Water contact angle, θ_W [°]	Average surface roughness, R_a [µm]	Polar component of SFE, γ^p [mJ/m^2]
SS 304 BB	59.1±2.2	6.46±0.27	12.86
SS 316 SP	59.5±4.2	0.29±0.01	10.35
SS 304 SP	63.0±5.0	0.43±0.01	7.03
SS 316 BB	70.6±8.6	6.69±0.29	6.9
PETG	85.0±2.5	0.22±0.04	1.36
PS	88.0±1.6	1.51±0.05	0.81

Bead blasted steels and polystyrene were characterised by rough structure whereas sand polished steels and PETG had smooth surface finish. All materials were divided into pairings of similar water contact angle/hydrophobicity. Analysis of biofilm development was performed for each pairing separately, to show the sole impact of substrate roughness.

4.4 Results and discussion

4.4.1 Attachment vs development: impact of roughness

The primary attachment of *Stichococcus* sp., *C. luteoviridis, H. pluvialis* and *C. Saccharophila* has been reported in Chapter 3.4.1 and presented in Figure 14. An ideal biofilm development would be represented by a high primary attachment followed by a high duplication rate and hence a high number of cells at the end of cultivation cycle. Initial adhesion of *Stichococcus* sp., *C. luteoviridis, H. pluvialis* and *C. saccharophila* was within the range 8.9±3.3 – 38.1±1.4 cells·10^5/cm^2. Most strains adhered in higher numbers to rougher materials (Figure 14). However, the initial colonisation did not vary significantly

between different materials. The effect of substrate structural characteristics was observed more on the quality of the biofilm deposition rather than the quantity of its attachment. On smoother materials, microalgae formed well-organised aggregates whereas on rougher ones, the biofilm matrix shaped around the structural characteristics of the substrate.

Table 11 Final biofilm density

Material	Final biofilm density, ρ [cells·10^5/cm^2]			
	H. pluvialis	*Stichococcus* sp.	*C. luteoviridis*	*C. saccharophila*
304 BB	22.7±5.9	10.4±4.7	110.2±11.2	8.2±2.7
316 SP	62.1±1.9	26.8±3.4	49.0±2.8	107.5±14.9
304 SP	18.0±3.7	21.1±1.5	88.0±2.7	28.9±4.8
316 BB	70.7±4.0	17.9±1.5	63.3±3.2	26.0±1.9
PETG	30.5±4.4	8.5±0.7	35.1±0.8	52.1±5.0
PS	42.1±1.5	9.2±2.7	46.0±1.7	59.4±2.4

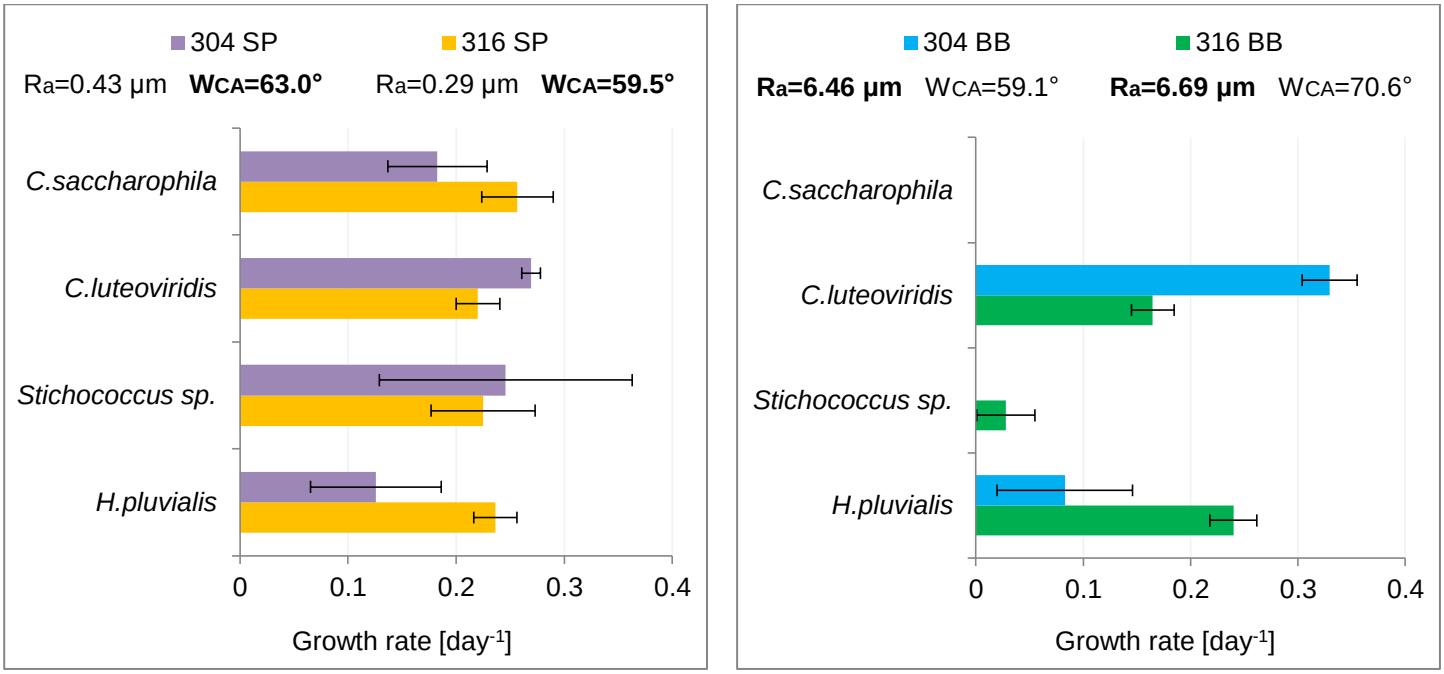
H. pluvialis, Stichococcus sp., *C. luteoviridis* and *C. saccharophila* were grown on stainless steels and plastics in humid atmosphere for 5 days. All measurements of biofilm density taken at 1st, 3rd and 5th day of cultivation are given in Appendix D.3. The final biofilm density, after 5 days of cultivation in aerial humid conditions, is presented in Table 11. To evaluate how material properties influence biofilm development, growth rates on particular materials were compared. Growth rates were chosen for comparison purposes instead of biofilm final density, as growth rates express the relation between final and initial density of biofilm.

4.4.2 Growth on steel materials

Two steel materials, 316 and 304, received different treatments and provided 4 substrates with very different characteristics in terms of roughness; hydrophobicity values also varied (from 59.1° to 70.6°) but not that significantly to be used for comparison (Table 10).

Data reported in Figure 24 illustrate the growth of the four microalgae on materials of similar hydrophobicity and composition, but varying average surface roughness. As mentioned before, microalgae attached in slightly higher numbers to rough substrates. However, the further development of biofilm was better on smooth steels. In general, microalgae grew faster on smooth steels (316 and 304 SP) in comparison to their rougher equivalents (316 and 304 BB) as it can be visible in Figure 24 A and B. The highest growth rates were obtained by *H. pluvialis* and *C. luteoviridis*: 0.24 and 0.33 day^{-1}, respectively.

Cells preferred to grow on smooth materials, which is clearly visible. *Stichococcus* sp. and *C. saccharophila* grew well on 316 SP (0.22 and 0.26 day^{-1}, respectively) in contrast to 316 BB where the number of cells in biofilm decreased over time.



A B

Figure 24 Growth rates at aerial conditions on smooth (A) and rough (B) steels

Growth rates of *C. luteoviridis* cells were also higher on 316 SP than 316 BB steel: 0.22 compared to 0.16 day^{-1}. The only exception was *H. pluvialis*, which grew at the same pace on both steels (r=0.24 day^{-1} on SP and BB steels grade 316). The growth rates on sand polished steel 316 were higher in comparison to rough 316 BB. Similar phenomenon was observed when the growth on smooth

and rough grade 304 was compared. Most of microbial biofilms grew faster on smooth substrate, with growth rates equal from 0.13 to 0.27 day^{-1}. On rough materials, biofilm developed slower: two microalgal species did not grow, whereas *H. pluvialis* grew at 0.09 day^{-1} growth rate. The only exception was *C. luteoviridis*. However, the growth of *C. luteoviridis* on smooth 304 SP substrate was only slightly lower than on rough 304 BB: 0.27 and 0.33 day^{-1}, respectively.

Analysis of roughness impact on biofilm development on 304 SP and 316 SP showed that even small difference in roughness can contribute to better growth of microalgae (Figure 24 A). Both materials had similar hydrophobicity, equal to 63 and 59.5°, respectively. The difference in average surface roughness was not significant: 0.43 compared to 0.29 µm. Smaller difference in roughness corresponded to lower differences between biofilm growth values, although most microalgae still preferred to grow on smoother 316 SP with 0.24-0.26 day^{-1} growth rates. On slightly rougher substrate (304 SP), microalgae biofilm developed with smaller growth rates, equal from 0.13 to 0.27 day^{-1}.

Grade 316 had higher primary colonisation and higher growth rates than in grade 304. It is an interesting phenomenon, since molybdenum that is contained within grade 316 supposed to inhibit further growth of microalgae. Biofilm inhibition took place only in the case of *C. luteoviridis*, which growth was lower on grade 316 than 304, despite similar primary attachment. At the same time, growth rates on sand polished steel were higher for almost all microalgal strains with only two exceptions: *C. luteoviridis* on 304 BB and *H. pluvialis* on 316 BB.

4.4.3 Growth on plastic materials

Growth of *Stichococcus* sp., *C. luteoviridis, H. pluvialis* and *C. saccharophila* on PETG and PS is presented in Figure 25.

C. saccharophila biofilm grew the best amongst tested microalgal strains on both PETG and PS. *Stichococcus* sp. did not grow well inside the reactor; the decline in growth rates was observable on both plastics. The number of cells

decreased from 3.3±0.4 and 1.8±0.3 to 0.9±0.3 and 0.9±0.1 cells·10^6/cm^2 on PS and PETG, respectively.

Final number of cells in the biofilms was very similar on PS and PETG for every tested strain (Table 11). However, when the value of primary attachment was taken into account, it turned out that the growth rates were higher on smoother PETG: 0.13, 0.14 and 0.19 day^{-1} compared to 0.08, 0.09 and 0.17 day^{-1} for *C. luteoviridis, H. pluvialis* and *C. saccharophila*, respectively (Figure 25).

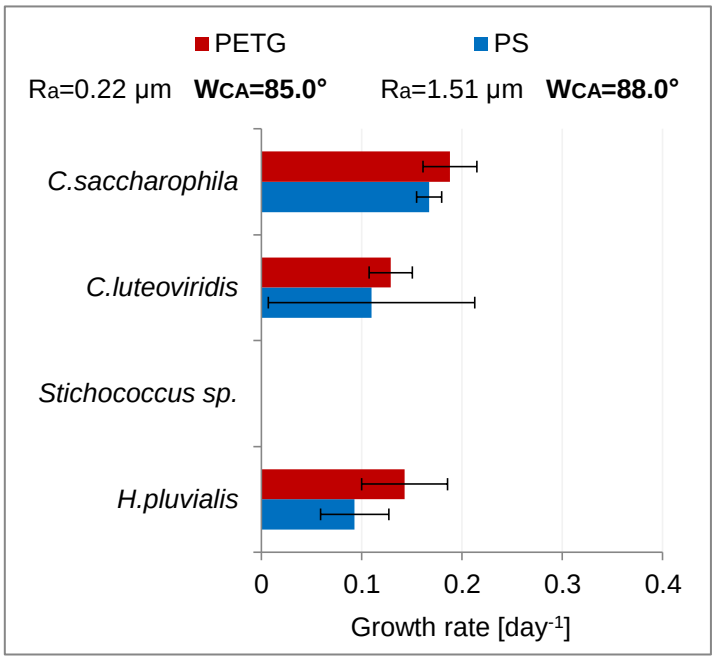


Figure 25 Growth rates at aerial conditions on PS and PETG with similar hydrophobicity but different roughness

Similar relation between primary adhesion and growth rates was observed on bead blasted and sand polished steel, where microalgae adhered more onto rougher substrate, but grew better on smooth material. An additional growth trial of *Stichococcus* sp. on the smoothest glass confirmed this suggestion. On glass, the number of *Stichococcus* sp. cells in the biofilm increased from 4.8 to 16.1 cells·10^5/cm^2 during 4 days of cultivation in HBBR. The final biofilm density was low in comparison to the best results obtained on other materials,

nevertheless the growth rate on glass was almost two times higher than the highest growth rates presented in this study (r= 0.40 day^{-1}).

4.4.4 Impact of surface topography on biofilm development

Primary attachment on bead blasted stainless steels was slightly higher than on sand polished stainless steels. Higher number of cells adhered to rough polystyrene than smooth polyethylene terephthalate. Material roughness was a surface feature that allowed cells to accumulate inside niches and adhere in higher numbers [248]. It agrees with the results presented in Chapter 3. It was suggested that the best materials for carrier substrate need to possess certain attachment points which would promote initial colonisation and protect cells from the influence of shear forces resulting from liquid movements [171]. Notwithstanding this, the value of average surface roughness was not correlated to the primary attachment suggesting that the surface structure is more important than numerical values of roughness [127].

With respect to biofilm growth, the comparison between rough and smooth substrates of similar hydrophobicity revealed that further multiplication of cells was promoted on smooth materials. Growth rates on sand polished steels were in general higher than growth rates on bead blasted steel, regardless the grade. Growth rates higher than 0.22 day^{-1} were obtained on almost all sand polished steels. Likewise, growth rates on bead blasted steels for three out of four microalgae (except *C. luteoviridis*) were below 0.10 day^{-1}. Similar results were achieved on plastics. Microalgae formed well- structured biofilms on smooth materials. Even though those biofilms consisted of smaller numbers of cells than on rough substrates, their organised structure allowed a more efficient nutrient transportation. Furthermore, cells were allowed to divide freely in any direction, whereas on rough substrates, cells were limited by structural features. Chung *et al.* [12] on their work on structured PDMSe suggested that the development of biofilm on rough substrates could be inhibited by topographical features of the material. The presence of structural obstacles limited the expansion of *S. aureus* cells and limited the biofilm's growth. In this chapter, increased cell accumulation in bead blasted steel niches contributed to the

formation of a less uniformly structured biofilm matrix which inhibited cells division and nutrient transportation.

4.4.5 Impact of physical properties of materials on biofilm development

The materials' polarity did not have an influence on the biofilm development of all tested microalgae. When all microalgal species were considered at the same time, the relationship was not straightforward and the linear relationship was very low with correlation coefficient equal to 0.48. Material polarity/biofilm growth dependence was visible only for *C. luteoviridis and C. saccharophila* with Pearson coefficient equal to 0.89 and 0.84, respectively, and t-test passed at 0.01 and 0.10 level of significance, respectively, indicating strong positive linear correlation (Figure 26 A). Growth of both *Chlorella* strains was also dependent on hydrophobicity of material, with Pearson coefficients equal to -0.90 and -0.72, indicating negative linear correlation (Figure 26 B). Part of the data on sloughed biofilms was not taken into account, due to possible disruption in results due to oversaturation.

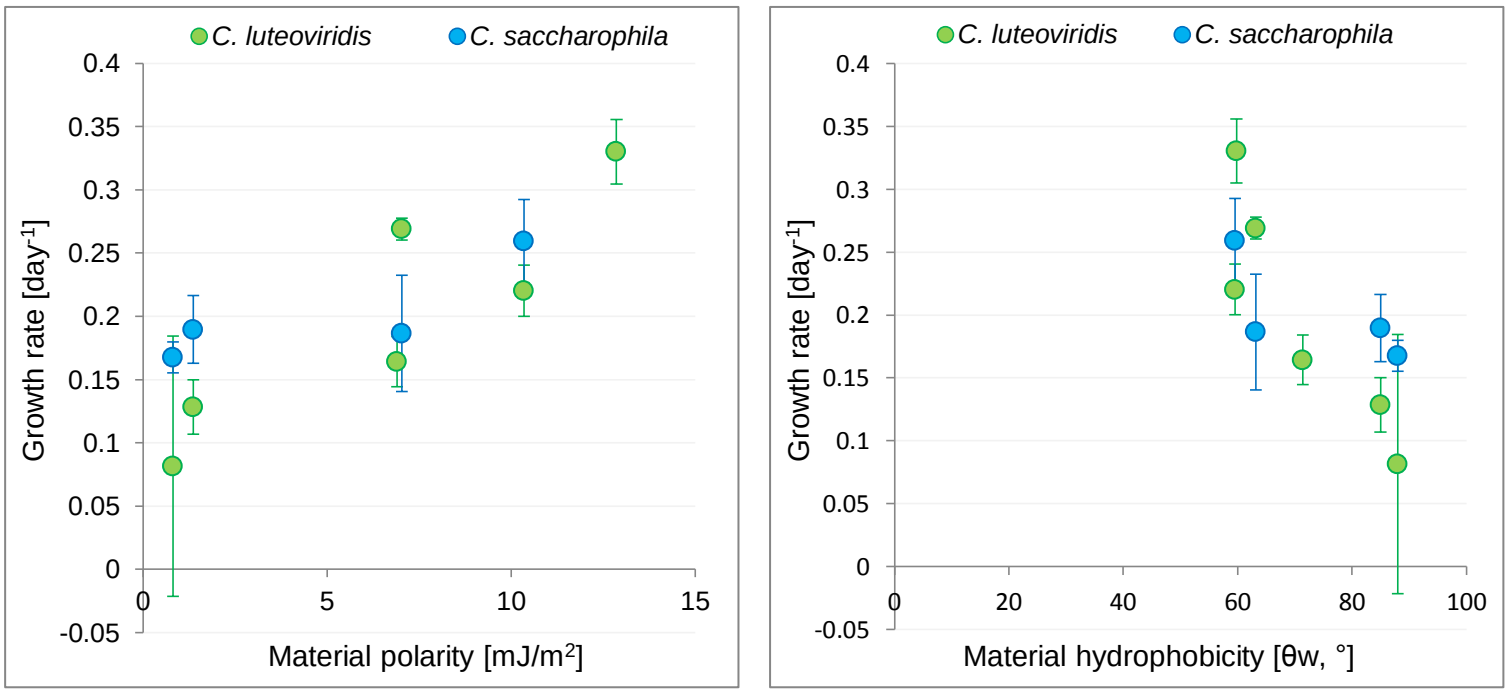


Figure 26 Growth rate of C*. luteoviridis* and *C. saccharophila* in relation to material polarity (A) and hydrophobicity (B)

T-test was not passed for the remaining strains, therefore the data showed no visible relation between polarity/hydrophobicity and biofilms' growth rate for *H. pluvialis* and *Stichococcus* sp. A study by Gomes *et al.* [240] on *E. coli* adhesion on silicone, stainless steel, polyvinyl chloride and glass showed that *E. coli* initial and final biofilm density was better on hydrophobic silicone. However, when growth rates were taken into account, it turned out that *E.coli* grew better on less hydrophobic substrates: 0.30, 0.27, 0.23 and 0.25 day^{-1} growth rates were obtained on substrates with 47°, 67°, 79.3° and 115.4° water contact angle, respectively. The same phenomenon was observed in this study for *Chlorella* strains. Therefore, it was possible that material's hydrophobicity has an influence on the biofilm development of some strains.

The presence of liquid medium influences the properties of substrate. Lorite *et al.* [53] showed that hydrophobicity of glass and silicone significantly decreases in the contact with Periwinkle wilt culture medium. After 3 days of contact, the hydrophobic surface of glass (θ_W = 82.2°) decreased below 10°. Silicon became hydrophilic even faster; only 3 hours of contact with medium were needed to drop from 59.4° to <10°. Appropriate experiments were performed in Chapter 5, in order to confirm whether the physicochemical properties of biofilm change over cultivation time. The hydrophobicity of biofilm changed over time which meant that surface hydrophobicity cannot influence further growth of biofilm. Nevertheless, experiments in this study showed that the initial hydrophobicity of the material can influence biofilm development. It is possible that hydrophobicity affected the structure and uniformity of the biofilm base, in a similar way as smooth materials promoted well-organised colonisation of cells. However, more experiments analysing the adhesion patterns of various microalgal strains on hydrophobic/hydrophilic materials would need to be performed to confirm this statement.

Araujo *et al.* [239] showed that bacterial biofilm adhered more to hydrophobic polypropylene than to hydrophilic glass; $4.6 \cdot 10^7$ cells/cm^2 compared to $8.2 \cdot 10^6$ cells/cm^2, respectively. However, final biofilm density was very similar on both materials, reaching $7.2 \cdot 10^8$ cells/cm^2 on plastic and 6.3 cells$\cdot 10^8/cm^2$ on glass

after 9 days of cultivation. Therefore, growth rates on hydrophilic glass were higher than on hydrophobic polypropylene: 0.54 compared to 0.34 day^{-1}, respectively. Araujo *et al.* suggested that the material's hydrophobicity did not favour further development of the microbial biofilm. The same assumption was made in a previous work by the same authors [249], where they tested *Methanosarcina barkeri, Methanosaeta concilii* and *Methanobacterium formicicum* bacterial attachment to substrates of different hydrophobicity and in the study of Lorite *et al.* [53], where they tested *Xylella fastidiosa* growth on glass and silicone. However, based on the experiments performed in this study and other studies [239] [240], it is clearly seen that material's hydrophobicity may influence biofilm development: some microbial species prefer to grow on more hydrophilic materials.

4.4.6 Impact of species selection on biofilm development

Most microalgal species tested in this study adapted to aerial conditions. The best growth was achieved by the *Chlorella* species: *C. saccharophila* and *C. luteoviridis*. The worst growing specie was *Stichococcus* sp. It was possible that *Stichococcus* sp. was not a suitable strain for cultivation in humid atmosphere, even though it is a natural environment for aeroterrestrial species growth. In contrast, another aeroterrestrial microalga *C. luteoviridis* grew very well on all materials inside the HBBR, therefore it cannot be concluded that aeroterrestrial species in general cannot be cultivated inside the novel reactor. Häubner *et al.* [80] suggested that the growth of *Stichococcus* sp. can be significantly inhibited at RH levels below 93%. The humidity oscillations could be an issue for *Stichococcus* sp. It may be caused by the lack of effective stress metabolites - polyols. It has been reported that the presence of polyols, such as ribitol, erythritol and sorbitol, have a protective function in microorganisms exposed to stress conditions and extreme environments. No trace of any polyol in *Stichococcus* sp. composition was suggested by Gustavs *et al.* [250]. They tested *Stichococcus* sp., *C. luteoviridis* and *C. saccharophila* for polyols and found that *Stichococcus* sp. cells lacked any of those compounds, whereas *C. luteoviridis* and *C. saccharophila* had 360±23 and 68±4 µmol/g of ribitol, respectively. Both *Chlorella* strains were more resistant to lower saturation

periods. To determine how the lack of polyols affected the growth of *Stichococcus* sp., a comparative study was conducted and presented in Chapter 5. The study was performed on *Stichococcus* sp.- aeroterrestrial microalgae without polyol and *C. saccharophila*- freshwater microalgae that contained ribitol. The work provides an insight on how *Stichococcus* sp. is able to grow in biofilm and how lack of polyols affected EPS excretion.

In contrast, aeroterrestrial microalga *C. luteoviridis* grew very well on all materials in humid atmosphere. It is possible that *C. luteoviridis* is able to sustain growth efficiently at lower relative humidity levels than *Stichococcus* sp. Bertsch [251] showed, that green aeroterrestrial microalgae *Apatococcus lobatus* can still uptake CO_2 at relative humidity levels above 68%, while 50% CO_2 uptake capacity was reached at 90% RH. Tolerance to lower humidity levels of *C. luteoviridis* is possible, as both *A. lobatus* and *C. luteoviridis* belong to the same *Chlorellaceae* family.

C. saccharophila is a freshwater microalga, mostly cultivated and researched in liquid environment. However, the strain was associated with subaerial surfaces in the British Isles [252]. It was also isolated from forest area in Collmberg [253]. *C. saccharophila* together with *H. pluvialis* were found among algal species isolated from tree bark, indicating that both species can be naturally found growing at terrestrial environment [253]. *H. pluvialis* was successfully tested in attached photobioreactors. Many studies had shown that this strain is capable of attachment to substrates and efficient growth in biofilms [254] [195] [255]. Therefore, it was suggested that *C. saccharophila* and *H. pluvialis* can grow at both freshwater and terrestrial habitats [256]. It could explain why those microalgae, commonly known as freshwater, were able to efficiently grow at aerial conditions.

4.4.6.1 Impact of species polarity on biofilm development

Polarity of microalgal species were proven to impact primary attachment in Chapter 3. As mentioned before, polar cells had to overcome higher repulsive forces to attach to substrates. Apparently, cell polarity also influenced further microalgal biofilm growth. As shown in Figure 27, *Stichococcus* sp. cells of

higher polarity multiplied slower than *C. luteoviridis* cells of lower polarity. Low polarity of cells tested in this chapter corresponded to the highest hydrophobicity, whereas the most polar cells of *Stichococcus* sp. were hydrophilic. Growth rates of less polar/ more hydrophobic cells were higher on all tested materials: 0.16, 0.22, 0.33, 0.27, 0.13 and 0.08 compared to 0.03, 0.22, 0, 0.22, 0, and 0 day^{-1} on 316 BB, 316 SP, 304 BB, 304 SP, PETG and PS materials, respectively.

In aquatic environment, hydrophobic cells could enhance recruitment of additional cells present in liquid medium by influencing hydrophobicity of biofilm. Negatively charged microalgae co-aggregate to hydrophobic surfaces, contributing to overall biofilm density. Cell-to-cell adhesion is improved for hydrophobic cells [257].

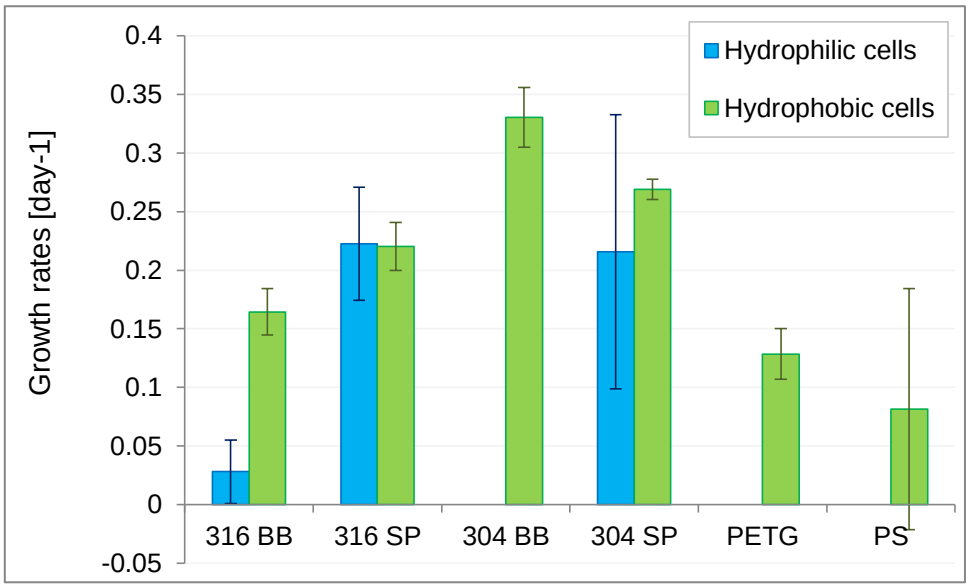


Figure 27 Biofilm growth rates of microalgae with high cell polarity (hydrophilic *Stichococcus* sp.) and low cell polarity (hydrophobic *C. luteoviridis*)

However, no free-floating cells are present in aerial conditions. Therefore, increased growth rates of hydrophobic/less polar cells are not a result of co-aggregation. It may be a result of increased metabolic pathways which allow more hydrophobic cells to degrade nitrogen and phosphorous [258]. More effective nutrient utilisation might contribute to higher growth rates of hydrophobic *C. luteoviridis* cells.

4.5 Conclusions

C. saccharophila, C. luteoviridis, Stichococcus sp. and *H. pluvialis* were able to efficiently grow in humid atmosphere with a maximum growth rate of 0.33 day^{-1}. Both freshwater and aeroterrestrial microalgal species survived in a non-aquatic reactor, although some species showed decreased growth rates. These lower values could be caused by varying conditions inside the reactor as relative humidity levels oscillated between 75-95% with temporary oversaturation periods. Hydrophobic *C. luteoviridis* microalgae were growing better than hydrophilic *Stichococcus* sp. on all tested materials, due to improved nutrient utilisation via metabolic pathways.

Microalgae indicated higher initial attachment to rougher substrates such as PS and bead blasted steels, although their growth was promoted on smoother materials such as PETG or sand polished steels. Cell colonisation on smoother substrates was slightly lower, although the biofilm formed was characterised by well-organised structure. Well-structured biofilm allowed a more efficient nutrient transport and did not limit further growth. On the other side, on rough materials, multiplication of cells was limited by the presence of substrate structural features; therefore lower growth rates were obtained. It seemed that material structure has a stronger impact on quality than quantity of attachment.

Hydrophobicity and polarity of materials significantly influenced *C. luteoviridis* and *C. saccharophila* biofilm development: growth rates were higher on less hydrophobic materials. Similar observations were made in other studies. However, no correlation between material's hydrophobicity/polarity and biofilm growth was found for remaining strains: *H. pluvialis* and *Stichococcus* sp.

5 Role of cellular EPS, SMP and surface topography of attachment substrate on biofilm development and strength

5.1 Abstract

The role of extracellular polymeric substances (EPS) and soluble microbial products (SMP) in biofilm formation and growth was investigated. Experiments were performed in aquatic environment on aeroterrestrial microalgae *Stichococcus* sp. and freshwater microalgae *Chlorella saccharophila* attached on two plastic materials: polystyrene (PS) and polyethylene terephthalate glycol-modified (PETG). Experimental data showed that the energy required for *C. saccharophila* cell removal decreases with decreasing SMP secretion. The same relation was shown for *Stichococcus* sp. biofilm, however only for mature biofilm. Therefore, a drop in SMP concentration weakened the biofilm matrix. EPS embedded microalgal cells and formed easily removable crust with progressing biofilm development. Secretion of EPS was suggested to be strongly specie-dependent.

5.2 Introduction

Algogenic organic matter (AOM) is often the cause of problems at water treatment plants, as it contributes to membrane fouling [259]. AOM is formed by algal cells, extracellular organic matter (EOM) and intracellular organic matter (IOM) [260] [261] [262]. EOM is released from algal cells by diffusion during the exponential growth phase and consists of low molecular weight metabolic intermediate (sugars, amino acids) also referred as soluble microbial products (SMP). IOM is released from senescent algal cells during cell lysis and is formed by high weight compounds (polysaccharides) [263] [264] [265] [266]. It is often referred to as extracellular polymeric substances (EPS).

Cell-surface attachment and biofilm development are complex processes depending on physiological (type of microorganisms), environmental (temperature, pH, and ionic strength of the media) factors and cell/substrata surface characteristics [267] [268]. Once developed, a biofilm structure will

include algal cells and IOM/EPS. This structure will provide an optimal environment for growth and survival [105].

Surface characteristics such as surface roughness, hydrophobicity and polarity can increase the extent of microbial colonisation. Characklis *et al.* [269] showed that the increase in microbial colonisation on rougher surfaces was linked to higher material surface area and a decrease in shear forces. Other researchers have established preferential attachment to hydrophobic, nonpolar surfaces such as plastics opposed to hydrophilic materials such as glass or metals [270] [271].

IOM/EPS composition is also very important in the attachment process. EPS composition has different species-related chemical and physical properties and it is mainly composed of polysaccharides, which determine the biofilm's primary conformation [272] [273]. Biofilm development and EPS production is also age- and space-related [274]. The initial attachment process (Cell-Surface) is provided by the adhesive polysaccharide-based EPS (Cell-EPS-Surface) [275]. Over time, the additional connector-molecules can be added to the EPS, changing the biofilm composition and the interactions between the two interfaces (secondary attachment). In many studies, extracellular polymeric substances excreted by microorganisms were isolated and analysed for their composition and function in microbial biofilm community [276] [277] [278] [279] [280] [281] [282] [283] [284]. Becker [285] reported that adhesion strength of certain diatoms is not always related to the amount of exopolymer produced, whereas other studies correlated the presence of EPS with propensity of microbial cells to attach [286]. Kotilainen *et al.* [287] tested 108 bacteria strains regarding their adhesion and EPS productivity, and showed that there was no connection between attachment and EPS expressed by amount of monosaccharide components. In 2000, Park *et al.* [286] tested attachment of activated sludge. Treated activated sludge sample had its EPS removed by both mechanical and chemical methods. The presence of EPS increased colony forming unit (CFU) per cm^2 of substrate from $175 \cdot 10^6$ and $125 \cdot 10^6$ to $330 \cdot 10^6$, for mechanical and chemical treatment, respectively.

Studies mentioned above are examples of investigations on EPS functions and its impact on primary attachment of cells. EPS is often referred as essential building component of biofilm. Nevertheless, there is a lack of studies that would prove its direct influence on strength of growing biofilm. There is also no study on the AOM effect on removal rates of biofilm. Therefore, this chapter investigated the role of surfaces microtopography, roughness and chemical properties of substrate on AOM production and strength of attachment of aeroterrestrial microalga *Stichococcus* sp. and freshwater microalga *Chlorella saccharophila* over time.

5.3 Materials and methods

5.3.1 Microalgal strains, materials and growth conditions

Cultures of aeroterrestrial microalga *Stichococcus* sp. and freshwater microalga *Chlorella saccharophila* were obtained from Culture of Algae and Protozoa, UK (CCAP 379/30 and CCAP 211/57, respectively). Those particular strains were selected based on study performed by Gustavs *et al.* [250]. They showed that *Stichococcus* sp. did not possess effective stress metabolites, polyols. At the same time, *C. saccharophila* contained 68±4 µmol/g of ribitiol. To illustrate how polyol presence in cells and the isolation habitat influence AOM secretion, *Stichococcus* sp. and *C. saccharophila* were chosen as model microalgae for investigations in this study. Cells were grown on liquid Bold Basal Medium at 22°C under continuous illumination, with white cool fluorescent lamps of 30µmol/m^2/s light intensity, in an Innova®44 orbital shaker (120 rpm) for 35 days.

Biofilm development was monitored on two different materials (3.5x3.5 cm): Polyethylene terephthalate (PETG - Plastic store Ltd, UK) and Polystyrene plastic (PS - VWR®). The average surface roughness measured by Olympus Lext confocal microscope was 0.22±0.04 µm and 1.51±0.05 µm for PETG and PS, respectively.

To promote microalgal attachment, PETG and PS were immersed in two reservoirs separately containing *Stichococcus* sp. and *C. saccharophila*.

Materials immersed in microalgal cultures were kept overnight at 22°C in darkness [244] [288] [289] to promote attachment. Initial concentrations of *Stichococcus* sp. and *C. saccharophila* were equal to 4.5±0.3 and 2.2±0.3 cells·10^6/cm^2, respectively.

After 16 hours of inoculation, samples covered with *Stichococcus* sp. and *C. saccharophila* biofilms were moved to two separate polypropylene reservoirs filled with BBM. Containers were kept under a fluorescent lamp with light intensity equal to 30 µmol/m^2/s at 12:12 illumination regime for 35 days. Two sacrificial samples for each material were collected and analysed at days 1, 4, 7, 10, 14, 21, 28 and 35.

Cell concentration (6 replicates for each sample) was measured by cell counting method using an Olympus CX41 optical microscope and Weber England haemocytometer (Improved Neubauer, Depth 0.1mm 1/400mm^2) according to Guillard and Sieracki [246].

5.3.2 Extraction of the algal organic matter

The extraction of AOM of *Stichococcus* sp. and *C. saccharophila* cells was performed using a modified method from Zhang *et al.* [290]. Each sample was removed from the reactor, rinsed briefly with buffer and placed in a 10 ml phosphate buffer solution [291] and sonicated using a Fisher Scientific FB 15051 ultrasonic bath at 37 kHz frequency. The 10 ml sample was centrifuged at 5000 RPM for 10 min using a Sorvall Legend RT+ bench scale centrifuge. The supernatant, containing the SMP fraction, was filtered through a Whatman® 0.45 µm membrane filter and analysed for protein and carbohydrate content. The pellets, containing the cells-bound EPS, were re-suspended in 10ml distilled water and placed on shaker for 10 min. To dissolve the cell-bound EPS, the samples were heated in the oven at 105°C for 60 minutes, cooled down to the room temperature and centrifuged for 10 minutes at 7500 RPM. After centrifugation, the supernatant was filtered through a Whatman® 0.45 µm membrane filter and analysed for protein and carbohydrate content.

5.3.3 Assessment of AOM protein and carbohydrate content

Total sugar and protein content of AOM were determined by colorimetric tests using glucose and bovine serum albumin (BSA) respectively as standards. Total sugar content was determined using the dinitrosalicylic colorimetric assay [292], protein content was determined using the Folin–Ciocalteu assay [293].

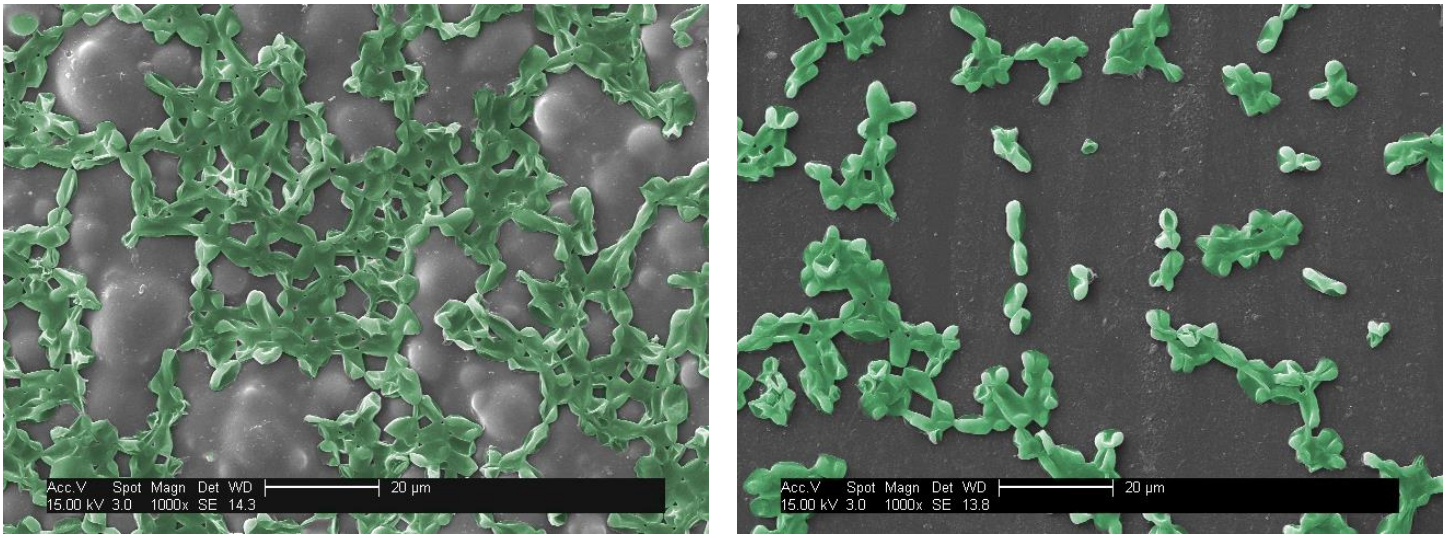
5.3.4 *Stichococcus* sp. attachment analysed by SEM

Field Emission Guns Scanning Electron Microscope Nova NanoSEM 630 (FEG SEM) was used to investigate how *Stichococcus* sp. adhered to PS and PETG materials. Evaluation of attachment patterns of these microalgae was especially important in order to observe how fast EPS is formed by polyol-free aeroterrestrial microalgae in contact with material surface. Concentration of EPS was too small to be measured at the beginning of cultivation, therefore it was essential to evaluate its secretion visually. Gimp 2.8.10 GNU Image Manipulation Program was used to mark *Stichococcus* sp. cells and EPS with green colour.

5.4 Results and discussion

5.4.1 SEM analysis of *Stichococcus* sp. attachment

The attachment of *Stichococcus* sp. cells to substrates was visually analysed by SEM (Figure 28).



A B

Figure 28 SEM images of *Stichococcus* sp. cells adhered to PS (A) and PETG (B), x1000. Cells and EPS marked by green colour

Visual investigations on *Stichococcus* sp. attachment revealed that these aeroterrestrial microalgae start to secrete small amounts of EPS at the very beginning of biofilm formation, approximately after 16 hours of inoculation. However, EPS concentrations during the first few days of cultivation were too small to be detected by the AOM quantification methods. Nevertheless, even small amount of EPS helped to form the structured aggregates of *Stichococcus* sp. that can be observed in Figure 28 A. Cells were visibly embedded and connected into a chain with EPS coating, which was also observed by Basak *et al.* [294] in their work on yeasts biofilm. The net of cells was present on both materials, although it had a more compact shape on PS. PS features allowed *Stichococcus* sp. to form a firmly attached biofilm base. Thanks to the gelling properties of EPS, cells could form certain attachment pattern utilizing unique features of PS. Cells accumulated around the polystyrene appendices. It is a well described phenomenon that surfaces' structural features help cell settlement and sustaining the initial biofilm formation. In addition, material features have been shown to influence the attachment pattern and shape of the microbial aggregates [38] [248]. Rougher substrates have also been reported to decrease shear forces [269] that usually contribute to biofilm sloughing [295]. In

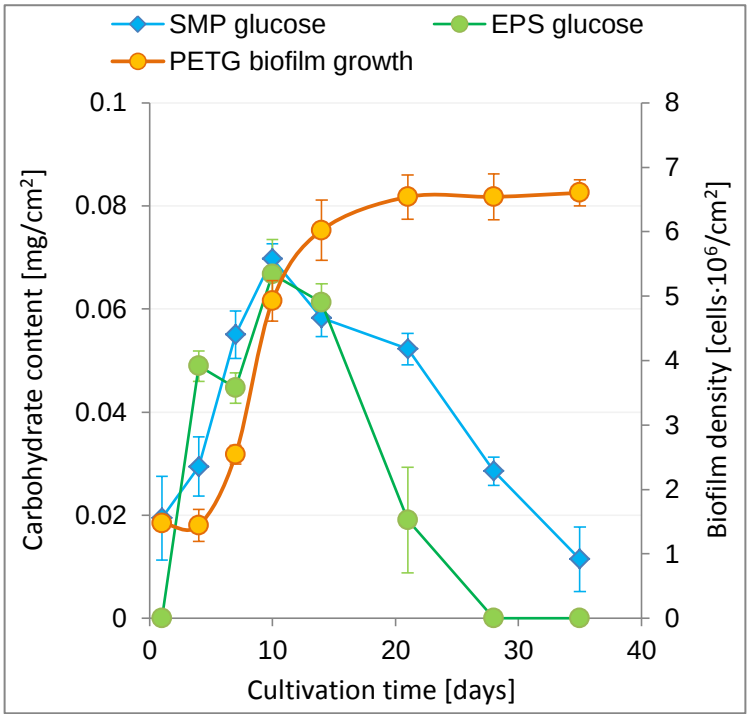
this case, it is suggested that PS structural features provided supporting function, protecting biofilm aggregates against the shear forces of the liquid flow. Detachment, which naturally occurs during microbial biofilm growth, is mostly dependent upon shear rates and microbial growth rates. It determines the biofilm shape and heterogeneity [296]. Therefore, the substrate shape and the presence of niches suitable for microbial attachment can help decrease detachment rate [295] and contribute to a more ordered and less random structure of the biofilm [297] [298]. *Stichococcus* sp. on PS showed a more structured biofilm (Figure 28 A). However, *Stichococcus* sp. cells were forced to facilitate in regard to the structure and characteristic features of the PS material, which could disturb formation of natural attachment patterns.

On PETG, *Stichococcus* sp. cells connected into long chains in order to maintain the biofilm support links and nutrient transportation (Figure 28 B). However, it was harder for cells to maintain the uniform shape due to lack of textural features in PETG structure. Therefore, the net of cells was broken at some places and lone cells were present. Chain breakage occurred due to moving liquid. Shear force affected the consistency of the biofilm matrix. Immature biofilm was not able to resist against shear forces on PETG; biofilm was too vulnerable due to small number of initially attached cells and small amounts of secreted EPS. However, the further development of biofilm was favoured on this smooth material more than on rough substrate, as shown in Chapter 4. To evaluate how microalgae indicated better growth on smooth materials, the following section described the secretion of AOM during 35 days growth and its effects on biofilm strength and growth.

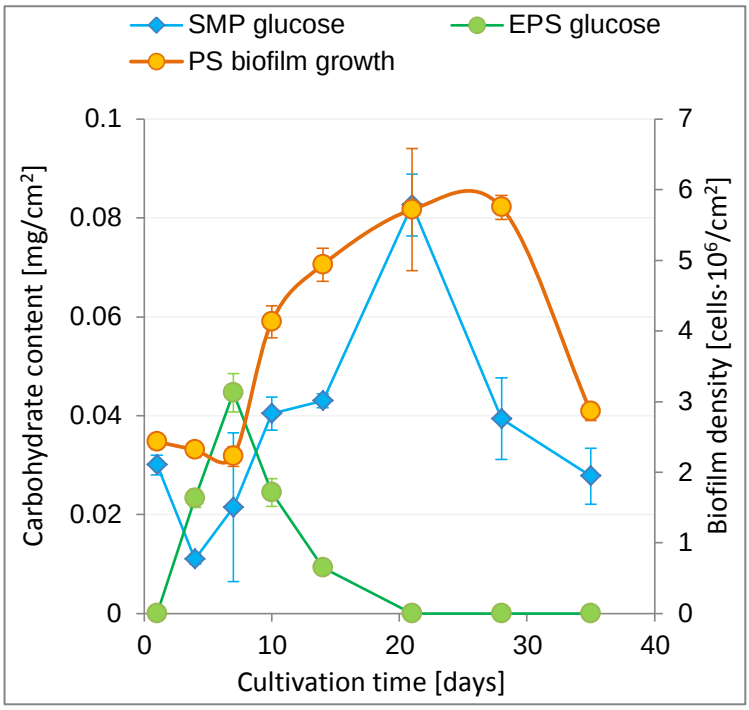
5.4.2 *Stichococcus* sp. biofilm growth and AOM secretion

Growth of *Stichococcus* sp. biofilm on PS and PETG over 35 days of cultivation is presented in Figure 29. The primary attachment, that is the attachment at 1st day of cultivation, of *Stichococcus* sp. was higher on PS than PETG with 2.4±0.1 and 1.5±0.1 cells·10^6/cm^2, respectively. Similar relation was showed in Chapter 3, where primary attachment of *Stichococcus* sp. on PS was almost two times higher than on PETG. Even though more cells adhered to PS, the

maximum growth rate was the same for both materials (0.21±0.03 day^{-1} compared to 0.22±0.03 day^{-1} for PS and PETG, respectively).



A PETG



B PS

Figure 29 *Stichococcus* sp. biofilm growth and AOM excretion

Growth of biofilm in this study followed microbial biofilm growth curve proposed by Bott (Figure 9, [23]). Exponential growth phase was reached by *Stichococcus* sp. after 5 days of lag phase on PETG and after 7 days on PS. Biofilm on PS and PETG entered a plateau phase around 20th day of cultivation. *Sichococcus* sp. on PS reached the end of growth cycle around 35th day of cultivation when the biofilm undergone sloughing, which was visible by a sudden drop in the number of cells (from 5.8±0.2 at 28th day to 2.9±0.1 cells·10^6/cm^2 at 35th day). At the same time, the growth cycle did not end on PETG yet, presumably because the structure of the formed biofilm was more uniform and contributed to longer duration of the whole matrix. Effective distribution of nutrients within well-structured biofilm on PETG allowed to maintain growth for longer than on PS. The maximum number of cells attached on PETG was 6.6±0.2 cells·10^6/cm^2, which means that at 35th day of cultivation the biofilm still did not reach sloughing phase. Nevertheless, the growth rates

were decreasing, which meant that the biofilm was close to the stationary phase.

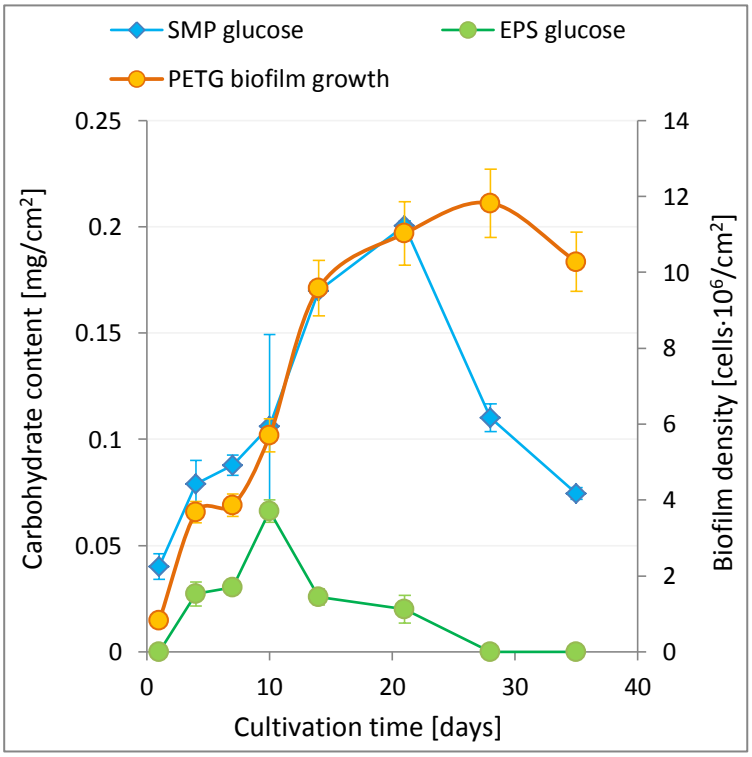
On PETG, the amount of EPS and SMP, expressed by carbohydrate concentration, was gradually increasing until the 10th day of cultivation, after which a drop in sugar content was visible. This drop was accompanied with biofilm entering decreasing rate of growth. On PS, the amount of SMP was increasing until 21st day of cultivation, when the biofilm entered plateau phase and cells started to die. Microalgae started to intensively secrete EPS at the end of the lag phase. At the exponential growth phase, cells were secreting more SMP than EPS. It was also suggested by Huang *et al.* [264], who stated that SMP are mostly excreted by diffusion at exponential growth phase.

Protein concentrations are given in Appendix D.5. Protein content in *Stichococcus* sp. did not change over the cultivation period. The differences in protein concentrations were not significant and did not result in any meaningful conclusions. As it was stated by Myklestad [299], carbohydrates can account for 80-90% of the total AOM concentration. It means that amounts of remaining compounds are very small and do not significantly contribute to overall AOM concentration. Therefore, after measurement of *Stichococcus* sp. protein concentration, it was decided not to perform such measurement for *C. saccharophila*, due to insignificance of those results. In Appendix D.5, the protein content of *Stichococcus* sp. was showed just for illustrative purposes, as the concentration did not change during the whole biofilm growth cycle.

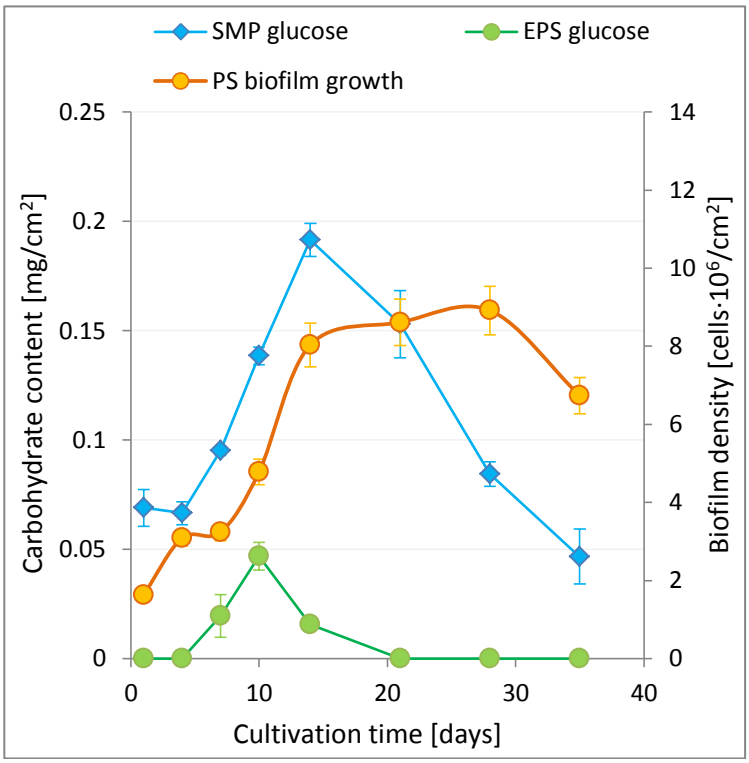
5.4.3 *C. saccharophila* biofilm growth and AOM secretion

Biofilm growth and AOM excretion by *Chlorella saccharophila* are presented in Figure 30. *C. saccharophila* attached in much lower numbers to PS and PETG materials, in comparison to *Stichococcus* sp. Only 8.3±0.7 cells·10^5 of *C. saccharophila* adhered to cm^2 of PETG in comparison to 14.8±1 cells·10^5 of *Stichococcus* sp. However, the initial amount of *C. saccharophila* inoculum culture was much lower than the density of *Stichococcus* sp. inoculum (2.5±0.3 and 4.5±0.3 cells·10^6 available per cm^2 of material, respectively). When the colonisation ratio is taken into account, around 53-64% of *C. saccharophila*

inoculum adhered to the substrate, while 41-68% of *Stichococcus* sp. inoculum attached onto the carriers. Both microalgal strains have very similar propensity to attach on given materials.



A PETG



B PS

Figure 30 *C. saccharophila* biofilm growth and AOM excretion

There was no visible lag phase in *C. saccharophila* growth. Cells started multiplication straight after inoculation. It may be caused by the fact that *C. saccharophila* was better suited against stress conditions, due to its ribitol content. Another reason behind quick assimilation is that this microalga can be grown at aeroterrestrial and freshwater environment, in both sessile and planktonic state. Exponential growth phase on both materials started at the beginning of cultivation and ended around 14th day of growth. However, *C. saccharophila* cells on PETG were still growing during the stationary phase, whereas on PS equilibrium between growth and detachment of cells was observed. Similarly as in the case of *Stichococcus* sp., structure of PETG promoted formation of well-established *C. saccharophila* biofilm. Organised structure of biofilm matrix contributed to efficient nutrient transportation and

higher protection against shear forces. As a result, *C. saccharophila* indicated better growth on PETG than on PS. *Chlorella saccharophila* attached in higher numbers to polystyrene substrate, although the further growth of this strain was promoted on PETG material. The growth rates on PETG and PS were equal 0.50±0.03 and 0.30±0.03 day^{-1}, respectively.

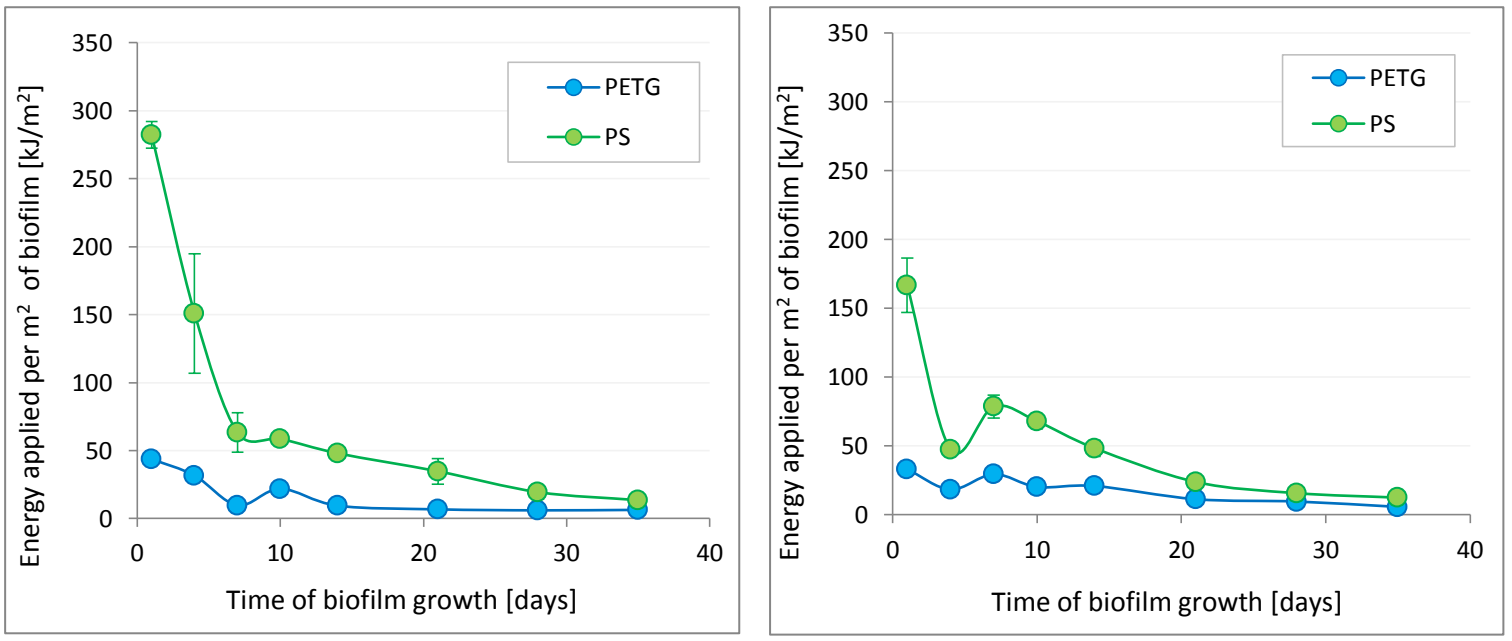
Similarly as *Stichococcus* sp. growth, *C. saccharophila* intensively secreted EPS at the end of lag phase. The highest productivity of SMP took place during exponential phase. As mentioned in previous section, similar phenomenon was observed in this study in *Stichococcus* sp. growth and by Huang *et al.* [264].

C. saccharophila reached sloughing phase around 35^{th} day of cultivation. The phenomenon of sloughing was observed on both materials.

5.4.4 Biofilm strength

As mentioned in section 5.2, the impact of AOM secretion on biofilm strength was not investigated yet. In this study, strength of biofilm was measured by detaching it from the substrate by sonication. Sonication is a more effective method for biofilm removal than scraping [300]. It allows detachment of the whole microbial community. It is important to supply appropriate level of sonication power and time, in order to remove active microbial cells. Microalgae have strong cell walls, although extraction of microalgal cells by sonication may result in cell disruption [301]. It is important to sonicate for a shorter period of time, to not break microalgae cell walls. Jeon *et al.* [301] showed that sonication at 40kHZ for 10 minutes does not result in effective cell lysis. Therefore, samples should not be constantly sonicated for more than 10 minutes. In their work on bacteria, Pierzo *et al.* [302] stated that the most optimum time for sonication is between 3.8-5.5 minutes. It was better for living organisms to be sonicated for a longer period of time at lower power level, rather than applying more powerful and faster sonication. In this study it was important to remove whole biofilm completely. Samples were sonicated until whole biofilm was removed, with 5 minutes break every 10 minutes, to allow rest of cells. Time of sonication was converted into energy that needed to be applied per m^2 of

biofilm in order to detach it. Energy applied for effective removal of *Stichococcus* sp. and *C. saccharophila* biofilms is presented in Figure 31.



A *Stichococcus* sp. **B** *C. saccharophila*

Figure 31 Energy applied to detach m^2 of microalgal biofilm

Primarily attached cells were very hard to remove from PS material. It took almost 30 minutes to sonicate them off the substrate, which consumed 282±10 kJ of energy per m^2 of *Stichococcus* sp. biofilm and 167±20kJ per m^2 of *C. saccharophila* biofilm. It was presumably caused by the structure of biofilm created on PS. Cells were firmly deposited between features of material; therefore it was hard to retrieve them. The net of cells deposited on PS was characterised by high uniformity, and it was harder to break the chain.

PETG had very low surface roughness and no features present, therefore it was easier to sonicate the biofilm off from the very beginning of cultivation. Shear forces of liquid medium that passes the biofilm contributed to sloughing [295]. Therefore, some of the chains were broken due to small medium movements. Only 44±5 kJ/m^2 was required to remove attached cells.

Energy required to remove cells from the substrates decreased with biofilm age, until it reached similar value for PETG and PS (~10 kJ/m^2). The only exception was a slight increase in detachment strength, observed for *Stichococcus* sp.

and *C. saccharophila* on both materials at the 10th and 7th day of cultivation, respectively. It is possible that the rapid increase in EPS secretion contributed to elevation in energy required for biofilm removal.

After some time of biofilm development, the presence of material features was no longer relevant. The biofilm thickness reached certain stage at which the whole biofilm acted as easily removable crust. EPS and SMP acted as glue connecting all cells; therefore it was easier to remove the whole biofilm with ultrasounds. This phenomenon was observed through the detaching process. When detachment was performed at the beginning of the cultivation, a small number of cells was dropping from the materials. If the time between attachment and detachment was longer, whole aggregates of cells were detaching from the carrier substrate. *Stichococcus* sp. and *C. saccharophila* cells while being removed from the substrates draw along other cells to which they were attached with EPS. Time required to detach biofilm was reciprocally correlated to the biofilm age for both microalgae.

5.4.5 Protein and carbohydrates as AOM indicators

The measurement of AOM concentration in this study was based on the conclusion that AOM are mostly composed of proteins [262] and carbohydrates [303] [304]. According to Myklestad [299], polymeric carbohydrates comprise around 80-90% of AOM. In 2008 Henderson *et al.* [304] showed that the amounts of carbohydrates in *Chlorella vulgaris, Microcystis aeruginosa, Asterionella farmosa* and *Melosira* sp. are always higher than amounts of protein. This relationship was found in both exponential and stationary biofilm growth phase, with the ratio protein: carbohydrate ranging from around 1:5 to 3:5 [304]. In this study, the average ratio of proteins to sugars for *Stichococcus* sp. was around 2:5 and was lower in stationary phase, similarly as for *C. vulgaris* biofilm in Henderson's work [304]. Proteins were detected at low concentrations in comparison to sugars. Similarly, in Wang's study on *Chlorella* sp. and *Micractinium* sp, protein concentrations in SMP reached no more than 0.02 mg/ml [305]. Furthermore, the initial concentration of proteins in SMP was also very low, around 0.01 mg/ml for both green microalgae [305]. In this study,

the initial protein concentrations were equal to 0.010 mg/cm^2 for SMP and around 0.007 mg/cm^2 for EPS, which was equivalent to 0.014 mg/ml and 0.008 mg/ml, respectively.

5.4.6 AOM excretion

The amount of excreted AOM was said to be strongly strain-dependent. In 2010, Henderson *et al.* [306] showed that microalgae in liquid culture can produce from 0.00095 ng to 0.65 ng of AOM per *Microcystis aeruginosa* and *Melosira* sp. cell, respectively. It meant that each strain is capable of producing different amounts of AOM. In this study, both strains secrete different amounts of AOM, depending on the material. *Stichococcus* sp. and *C. saccharophila* could excrete up to 0.045 and 0.040 ng of AOM per cell on PETG. On PS, *Stichococcus* sp. secreted maximum of 0.024 ng/cell, whereas *C. saccharophila* was able to excrete 0.035 ng/cell. It illustrates that AOM secretion is not only specie-, but also substrate-dependent.

5.4.7 SMP excretion

High amounts of SMP were produced strictly after microbial attachment. SMP acts as chelating agent [307], which means that it helps in bonds formation with metal ion. Chelators also form complexes with other substrates, therefore the SMP helped biofilm community in surface conditioning. The SMP accumulation on material surface was very straightforward [111]. It mechanically supported cell adhesion by forming filamentous matrix [308]. The SMP production was associated with microbial growth, which was clearly visible in the case of *C. saccharophila* and *Stichococcus* sp. Increase in SMP concentration was coupled with increase in cell concentration on both PETG and PS. Utilisation associated products (UAP) are SMPs excreted via substrate metabolism and biomass growth [309]. *Stichococcus* sp. and *C. saccharophila* produced more UAP than non-growth associated SMP. Biomass associated products (BAP) were produced when cells undergo endogenous decay [309]. Cell lysis and biomass decay contributed to BAP extraction. When microalgae reached stationary phase followed by sloughing phase, SMP production dropped. It means that microalgae excreted more UAP than BAP, and overall SMP

productivity was the highest during the exponential growth phase. The only exception was SMP excretion by *Stichococcus* sp. on PS (Figure 29 B). On this material, more carbohydrates were produced by microalgae during stationary growth phase than in exponential growth phase. Similar trend was observed in the study of Widrig *et al.* [310].

Wang *et al.* [305] observed that the highest amount of SMP was excreted by *Chlorella* sp. and *Micractinium* sp. at second week of cultivation. Even though the work performed by Wang was on planktonic state, some similarities with microalgae grown in a biofilm environment can be found. In this study, both *C. saccharophila* and *Stichococcus* sp. produced high amounts of SMP around the second week of progressive biofilm growth. In Wang *et al.* [305] work, after four weeks of biofilm growth, concentration of SMP decreased for *Chlorella* sp. *Micractinium* sp. completely stopped secretion of SMP at 31st day of cultivation. This study had confirmed the decline in SMP production. Around the third week of cultivation, smaller amounts of SMP were secreted by both microalgal strains.

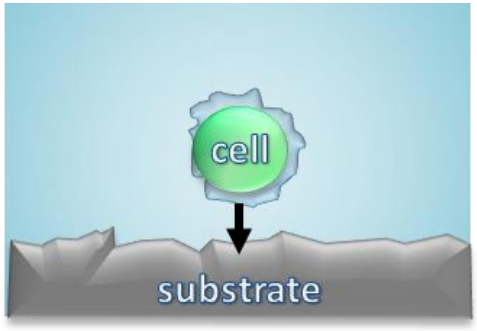
5.4.8 EPS excretion

SMP promoted cell deposition during initial biofilm formation [105] [106]. However, the further development of complex matrix was dependent upon EPS production. Negligible amounts of EPS were produced by microalgae until the 4th day of biofilm cultivation - cells did not excrete much EPS during reversible attachment. Similar behaviour was observed by Allison and Sutherland [311]; S61 and R1a freshwater bacterial strains synthesize EPS only after the adhesion took place. When primary attachment ended, the consolidation of the whole biofilm took place; more complex matrix of irreversibly attached microorganisms was formed [312]. It was visible by enhanced production of EPS and end of lag phase. At the beginning of the exponential growth phase, cells intensively produced EPS. Therefore, cell adhesion was improved by a large amounts of EPS via polymeric interactions [313]. Moreover, EPS was able to assist in adhesion and co-aggregation. It was proved that gram positive bacteria produce EPS to form colonisation bridges between cell and substrate,

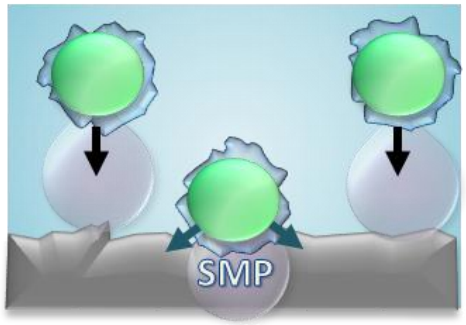
in order to deposit onto material [314]. Therefore, it is possible that the amount of secreted EPS was connected to the number of attached cells because they promote the adhesion.

5.4.9 SMP and EPS function in biofilm growth

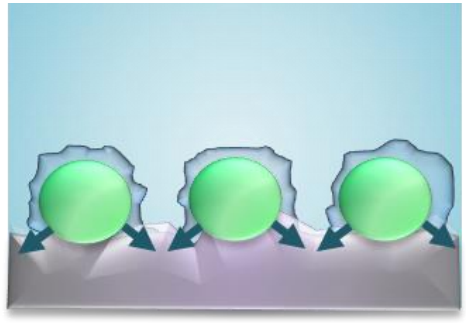
The schematic diagram of biofilm growth, SMP and EPS excretion by microalgae is given in Figure 32. Electrical and van der Waals forces together with polymeric interactions influenced microbial attachment [315]. Quorum sensing allows microalgae to identify attachment surface they come in contact with. Small signal molecules allow microalgae to communicate between cells and help in colonisation process [316]. Acyl homoserine lactone can be an example of small molecule that regulates processes associated with biofilm deposition and growth [317]. After first contact between cell and substrate surface, so-called reversible attachment occurs. It can be either intermediate state leading to biofilm formation (sequential theory) or a state coexisting with irreversible attachment (competitive theory) [318]. Either way, the terminology of reversible and irreversible microbial adhesion is still not understood [319], as a vast majority of 'irreversibly' adhered cells undergo self-triggered detachment during sloughing phase. In this thesis, the term 'reversible' will describe attachment of cells that came in contact with substrate surface, whereas 'irreversible' will describe the attachment of cells that already grow in biofilm community. Strong biofilm base was formed with the help of SMP conditioning layer and EPS that bond cells together. However, as soon as biofilm base was established (irreversible attachment), microbial community focused more on cell multiplication rather than EPS excretion. Kuhne *et al.* [320] stated that EPS excretion decreases with increase in growth rate, which was also observed in this study. It means that microalgae secrete EPS to a certain extent, at which community development is a primary concern.



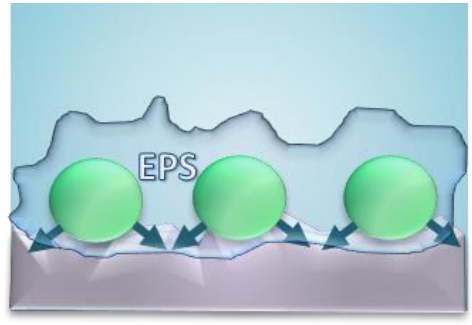
Day 0
Microalgal cell approach substrate surface



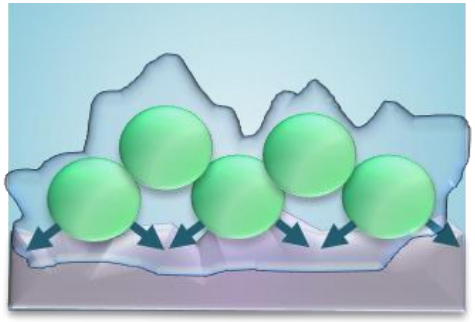
Day 1
Cells secrete SMP to form condition layer on substrate



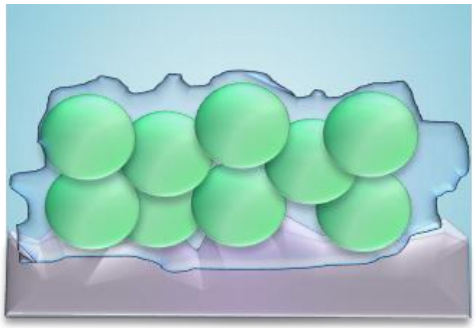
Day 4
Microalgal cell reversibly attach to the surface



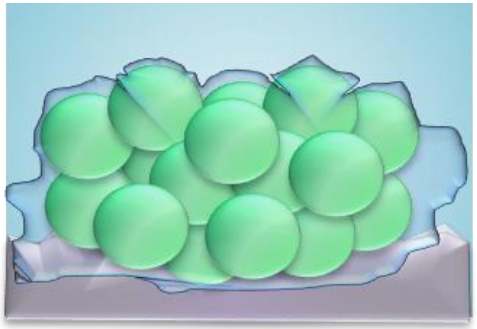
Day 7
Cells start to extensively produce EPS



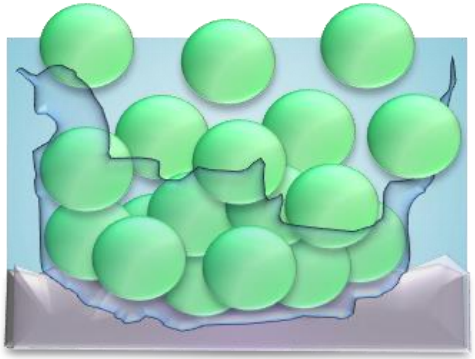
Day 14
EPS secretion rates decrease, cell multiplication intensifies



Day 21
Biofilm matrix forms, irreversible attachment takes place



Day 28
Biofilm density reaches peak, EPS start to degrade



Day 35
EPS break, cells move to deposit on new nutrient-rich environment

Figure 32 Biofilm growth process

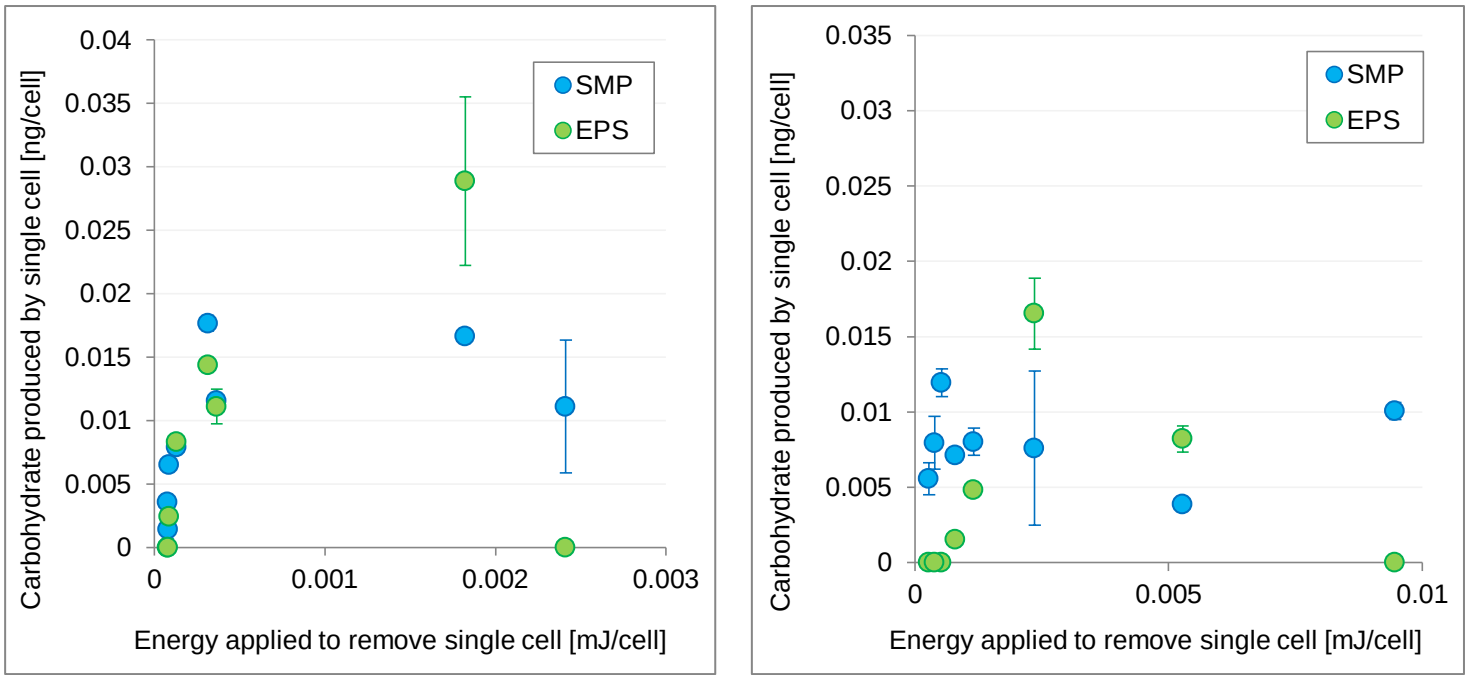
At the end of growth cycle, biofilm undergoes sloughing. EPS break and release cells, allowing colonisation of another surface. Sloughing of biofilm is caused by several factors. First, and probably the most important, is the lack of nutrients. When biofilm community suffers from insufficient amount of nutrients, cells are not able to multiply and grow. Therefore, it is important that cells could be released from biofilm community in search for nutrient rich environment they could colonise. Natural detachment of cells is triggered by quorum sensing, as suggested by Rice *et al*. [320] in their work on *Serratia marcescens*. Another factor that induces biofilm sloughing can be oxygen depletion [101]. When in-depth availability of oxygen in biofilm drops, large portions of biofilm detach. Sloughing in denitrifying biofilms can be triggered by formation of nitrogen bubbles [321]. Moreover, shear forces of moving liquid have an influence on biofilm detachment. Liquid medium moving with high velocity exert higher shear forces on biofilm, leading to cell removal [135].

5.4.10 Influence of AOM production on biofilm removal

5.4.10.1 Removal of Stichococcus sp. *biofilm on PETG and PS and AOM excretion*

In Figure 33 A the amount of energy applied to remove a single cell of *Stichococcus* sp. from PETG together with amount of SMP and EPS excreted by this cell was given. It was not possible to connect rapid increase in EPS and SMP production with biofilm strength. Initial colonisation required more energy to be sonicated off the substrate, even though the concentrations of AOM were low. Therefore, it can be concluded that presence of AOM did not alter the strength of initial biofilm. Energy applied to detach cell drops significantly for already established biofilm starting from day 4. The same trend was observed in EPS and SMP production, which also decreases after 4th day of cultivation. It may seem that AOM have an influence on the strength of mature biofilm. However, it is rather the decrease in EPS and SMP concentration that made the

biofilm weaker and therefore easier to detach. Only the drop in AOM concentration influenced the force required to remove biofilm; its production did not strengthen the biofilm. In fact, the biofilm strength was more dependent upon surface characteristics and biofilm age, more than AOM excretion. The unique rough texture of PS required more force to detach a cell than PETG as it can be seen in Figure 33 B.



A PETG **B** PS

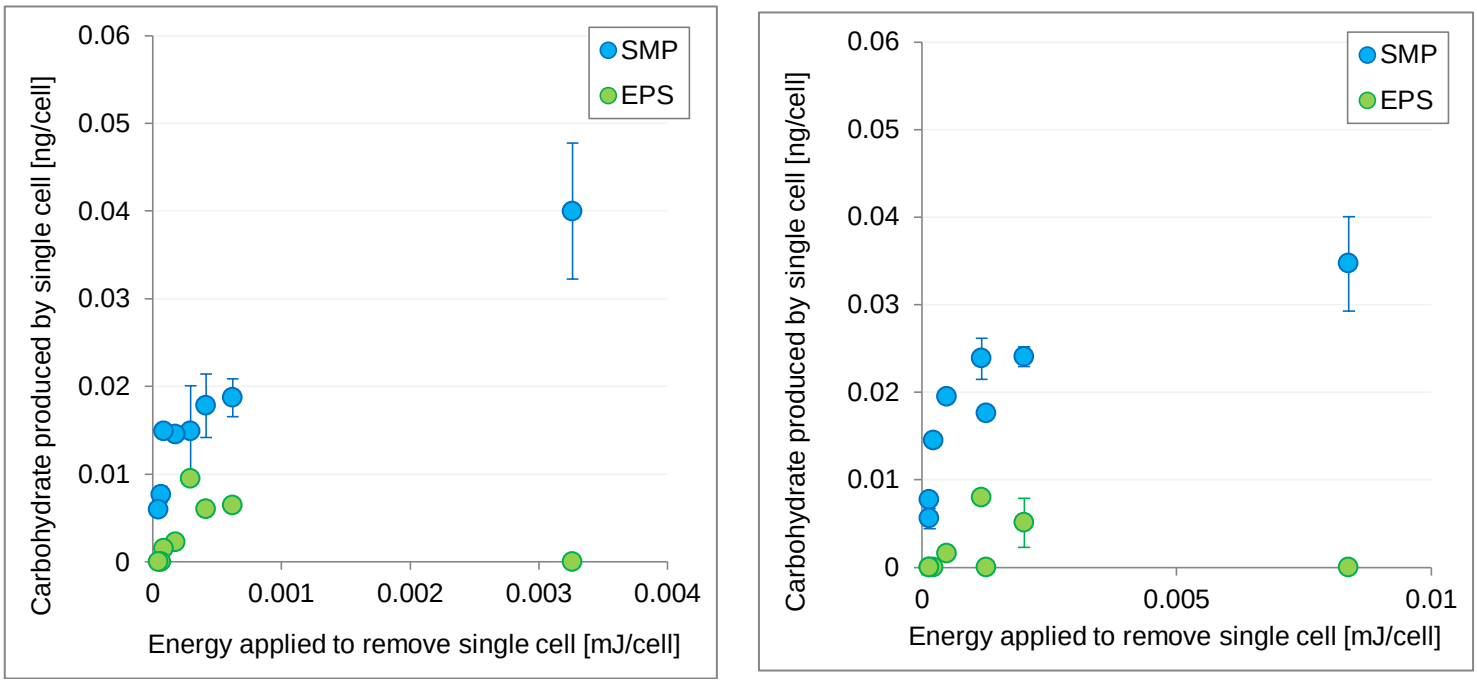
Figure 33 AOM excretion by single *Stichococcus* sp. cell grown and energy applied for cell removal

Similarly no straightforward correlation between AOM excretion and initial biofilm strength was observed on PS material, as shown in Figure 33 B. When biofilm was still at lag phase, AOM excretion did not influence its strength. However, for mature biofilm, decrease in EPS production influenced strength of the whole microalgal matrix. When biofilm reach so-called 'locking phase', it excreted more EPS and enclosed the whole microalgal matrix [323]. Irreversible adhesion was coupled with increased complexity of biofilm structure; starting from this moment, biofilm was regarded as 'mature' [312]. This phase of growth was very rapid and the community reached the EPS peak on PS at first week of cultivation. Starting from 7th day, when cells entered the exponential growth

phase, concentration of EPS was dropping together with energy applied to remove cell.

5.4.10.2 Removal of C. saccharophila biofilm on PETG and PS and AOM excretion

In the case of *Chlorella saccharophila* biofilm removal, the influence of EPS and SMP excretion on biofilm strength was visible. Data are presented in the Figure 34.



A PETG **B** PS

Figure 34 AOM excretion by single *C. saccharophila* cell grown and energy applied for cell removal

Energy applied to remove single *C. saccharophila* cell was strongly correlated to the amount of AOM excreted. The decrease in removal energy was coupled with the decrease in SMP concentration in the culture. In addition, the sudden increase in SMP excretion at 7th day of biofilm growth was also accompanied with increase in detachment energy. Therefore, it can be concluded that EPS and SMP have an influence on *C. saccharophila* biofilm strength.

5.4.11 Influence of surface properties on AOM excretion

5.4.11.1 Differences between PETG and PS

Before the impact of surface properties on AOM excretion can be analysed, the differences between tested materials need to be investigated. Polystyrene and glycol-modified polyethylene terephthalate are both thermoplastic materials. They have similar density (1.3 and 1.0 g/cm^3 for PETG and PS, respectively) and strength (41 kN-m/kg for PETG compared to 32-44 kN-m/kg for PS) [227] [324]. The differences between PS and PETG arise from their chemical composition and texture. Both plastics are built up from carbon chains; however PETG contains additional oxygen $(C_{10}H_8O_4)_n$ while PS consists of styrene chains $(C_8H_8)_n$.

PETG in appearance is transparent material characterised by high clarity. Its finish is smoother than PS; the average surface roughness of PETG used in this thesis was equal to 0.22±0.04 µm. It was much lower in comparison to average surface roughness of PS, which was measured to be equal to 1.51±0.05 µm. Difference in roughness was clearly visible in materials structure. Even though surface roughness was proven not to have a direct influence on microbial attachment (Chapter 3) [325], surface irregularities may have an impact on biofilm deposition [171]. PETG possess no visible textural features (**Error! Reference source not found.**), whereas PS consists of many contact points in the form of round appendices (**Error! Reference source not found.**).

5.4.11.2 Surface topography influence EPS excretion

The type of material had an effect on SMP and EPS excretion by *Stichococcus* sp. It seemed that the amount of EPS produced by microalgae was strongly connected to the surface of the material. Both aeroterrestrial and freshwater strains excreted more EPS and SMP on smooth PETG than on rough PS, as it is shown in Figure 35.

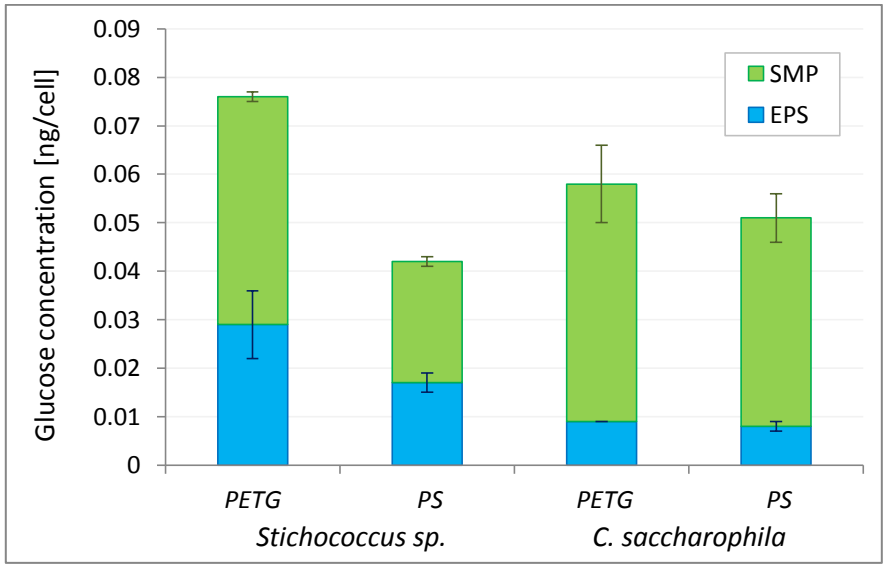


Figure 35 Maximum AOM secretion by *Stichococcus* sp. and *C. saccharophila*

The maximum amount of EPS produced by *Stichococcus* sp. and *C. saccharophila* on PETG was 0.029±0.007 and 0.009±0.000 ng/cell respectively, in comparison to 0.017±0.002 and 0.008±0.001 ng/cell on PS. Characteristic topography of PS material forced microalgal cells to deposit along its features [297] [298], as shown in Figure 36. Attachment on PS was less random, as cells were forced to colonise along material convexities. More cell-to-substratum interactions took place and cell-to-cell communication was limited. Smooth surface of PETG resulted in less random structure of biofilm, as cell attachment was not dependent upon certain surface characteristics. The schematic diagram of cell attachment on smooth surface is presented in Figure 37. More ordered arrangement of microalgal cells offered more possibilities of cell-to-cell interactions that enhanced microbial communication and lead to increase in EPS production [313]. Furthermore, biofilm formed on PETG due to material structure suffered from increased influence on shear forces that contributed to natural detachment. Under such conditions, biofilm tended to excrete more EPS [326]. The reason behind it is that biofilm tent to overcome forces connected with medium movements and therefore stabilise the structure of whole community [327].

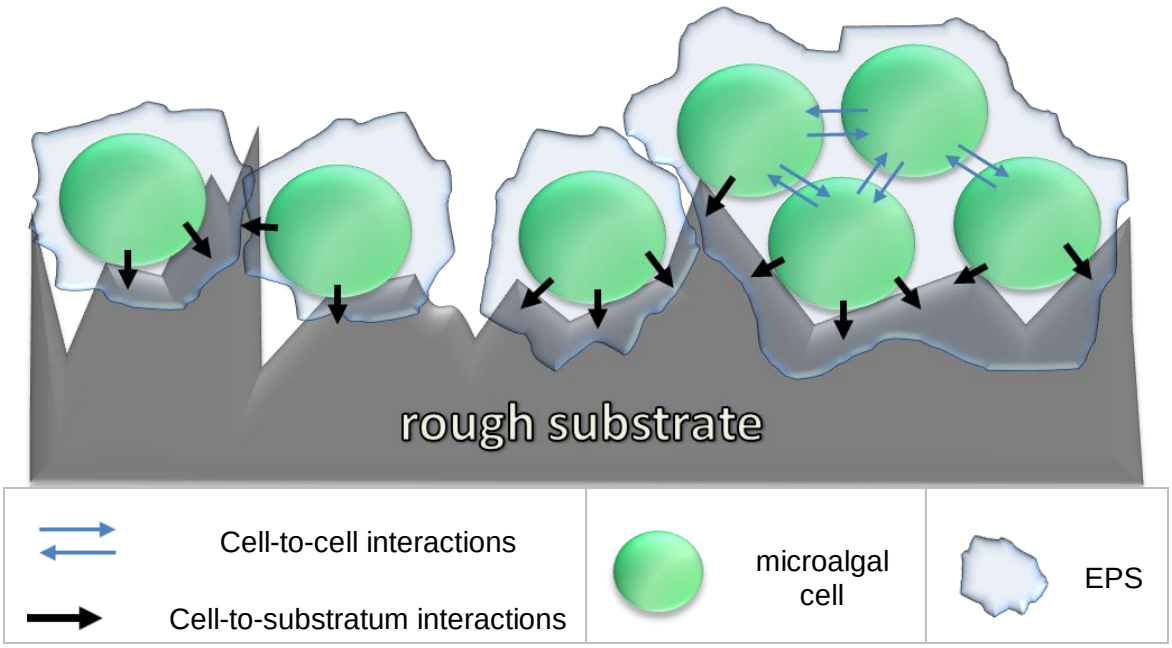


Figure 36 Microalgal cell attachment to rough substrate

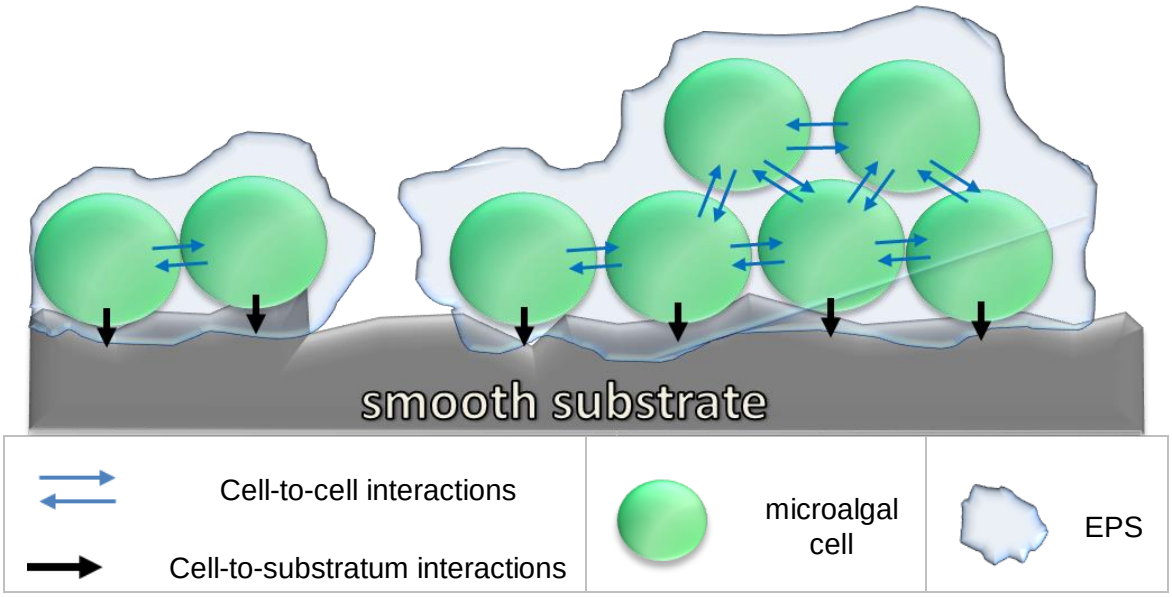


Figure 37 Microalgal cell attachment to smooth substrate

5.4.11.3 Chemical properties of PETG influence EPS excretion

Most algae are obligated aerobes, which means that they require oxygen to grow [328]. Microalgae synthesize complex carbohydrates and oxygen from CO_2. Part of produced oxygen is consumed by microalgae via cellular respiration and converted into energy. The common problem associated with polyesters such as PETG is hydroxide and peroxide groups build-up on the

surface of plastic. Those groups can be a source of oxygen radicals. Presence of oxygen in PETG carbon chains explains why both *Stichococcus* sp. and *C. saccharophila* excreted more EPS on this material. In their work on bacterial social interactions, Blanchard and Lu [15] referred to cellular interactions of *Pseudomonas fluorescens*. This bacterial strain tends to secrete more extracellular polysaccharide in order to access oxygen [329] [330]. Oxygen regulates biofilm growth, as its depletion is a common problem in standing cultures [331]. Overproduction of EPS result in improved cycling of elements throughout biofilm [152], therefore microalgal consortium has an improved access to oxygen source on PETG.

5.4.12 Influence of cultivation environment on AOM secretion

In this chapter, tests on AOM secretion were performed in aquatic environment. Concentration of EPS could not be measured in humid atmosphere, as self-induced detachment of EPS could interfere with the results. In aquatic environment, broken EPS can enter liquid medium and is not present in biofilm. Therefore concentration of EPS measured in aquatic environment reflected its formation and degradation accurately. On the contrary, detached EPS would stay on the sample in humid environment as they would have no means of escaping into the medium. Consequently, measured concentration of EPS of biofilm grown in humid conditions would not reflect the phenomenon of self-detachment and would not accurately indicate EPS productivity. Study in this chapter and other studies [332] [333] suggest that after certain time EPS degrade and their productivity decreases over time. Jiao *et al.* [334] measured concentrations of EPS at aerial conditions for mid-development stage and mature biofilm. They showed that amount of EPS was two times higher in mature biofilm; 340 mg of EPS per g of dry biofilm weight compared to 150 mg/g. It means that measurement of EPS concentration in sub-aerial biofilm will not show decrease in their production rates, but rather indicate the amount of EPS produced over the whole duration of biofilm growth.

There is no study on changing EPS concentration over cultivation time. EPS productivity rates in humid atmosphere are not known, although it can be

concluded that microorganisms are secreting EPS in similar manner as in aquatic environment [335]. Microorganisms were proven to produce EPS even in aerial environments [336] [337]. EPS secreted by biofilms in humid conditions have similar functions as substances secreted by biofilms in aquatic environment: EPS protect against stress conditions, enhance cellular growth and support structural matrix of biofilm. Apart from those functions, EPS help aerial biofilms in maintaining growth at lower humidity levels. EPS entrap and retain water [338] to sustain metabolic activity at lowest concentrations of water [339]. As a result, due to humidity oscillations, sub-aerial biofilms usually do not grow at their full metabolic potential [340].

At the beginning of the biofilm growth in humid atmosphere, significant amounts of SMP and trace amounts of EPS were already present in the biofilm after inoculation. However, it is not known how much EPS was secreted in humid atmosphere. There is no study on AOM secretion by aeroterrestrial microalgae at aerial conditions over cultivation time. As it was shown in this chapter, *Stichococcus* sp. required high concentrations of EPS in order to sustain its growth in aquatic environment due to lack of polyols (Figure 35). Therefore, its decline in growth in humid atmosphere, presented in Figure 37, may be a result of limited secretion of EPS due to low metabolic activity in aerial conditions. At the same time, *C. saccharophila* secreted much smaller amounts of EPS in aquatic environment (Figure 35). *C. saccharophila* grew well in both environments, with highest growth rates on smooth PETG than rough PS (Figure 37). It is possible that this microalga did not require high quantities of EPS in order to maintain growth, as it already contained stress effective metabolite, ribitol [250].

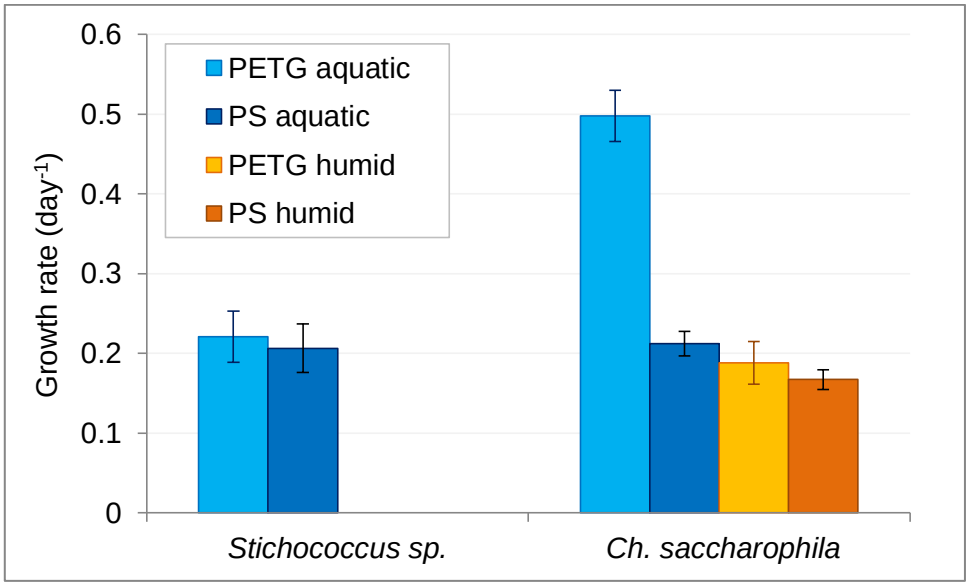


Figure 38 Growth of biofilm in aquatic and humid environment

Structure of material did not play a significant role in development of *Stichococcus* sp. biofilm in aquatic environment. Microalgae grew in the same pace on rough PS and smooth PETG, as shown in Figure 38. Similar growth rates were indicated by *C. saccharophila* on PETG and PS in humid atmosphere. However, in both cases the growth on smoother PETG was slightly better, when errors are not considered. For *C. saccharophila* in aquatic environment, the difference between growth rates on PETG and PS was more visible.

5.5 Conclusions

Algogenic organic matter is important in biofilm formation and development. SMP production enhanced primary attachment by conditioning the substrate, while EPS allowed formation of more complex and consolidated matrix of cells.

The initial colonisation on PS was more uniform than on PETG. The presence of multiple attachment points on PS enabled *Stichococcus* sp. to attach in higher numbers. It was easier to break aggregation chain on PETG due to the lack of protective features and exposition to detachment forces resulting from medium flow. However, the increased secretion of EPS on PETG after 4th day of cultivation enabled cells to maintain uniform growth. The growth of biofilm on

both PETG and PS was very similar and followed lag, exponential, stationary and declining rate growth phases, characteristic for biofilm growth. The highest amounts of SMP were produced during exponential growth phase, as indicated by other studies. The most EPS was produced at the end of lag phase, when cells start irreversible attachment. No EPS was secreted during sloughing phase, as it was reported by other scientists.

Strength of biofilm was evaluated based on measuring the energy required to detach biofilm. In general, biofilm removal from PETG took much less energy than removing biofilm from PS. It was caused by difference in structure between smooth PETG and rough PS. PS has average roughness almost seven times larger than average roughness of PETG (1.51±0.05μm in comparison with 0.22±0.04μm, respectively). Therefore, structural appendices on PS disturbed the removal of cells, whereas smooth surface of PETG contributed to easier detachment of the whole biofilm. Moreover, structure of material contributed to formation of well-organised biofilm on PETG, which was much easier to be sonicated at once. On PS, cells were forced to deposit along characteristic structural features. Biofilm matrix was strongly embedded with material structural features, making it harder to detach biofilm on PS. Furthermore, removal was influenced by decreasing rate of EPS production. Mature biofilm was weakened by declining EPS excretion, therefore the removal energy for dying biofilm was the lowest for all microalgal-substrate pairings.

6 Prediction tools for attachment and biofilm growth

6.1 Abstract

Microalgal cell primary attachment tests and initial biofilm growth trials are time and cost consuming, hence the need for an effective and simple model for the prediction of microalgal adhesion and development on new surfaces. The thermodynamic (TD) model, widely used for primary attachment prediction in bacterial biofilms, was analysed and applied in a wide study including 36 material-microalgae pairings. The TD - Work of attachment (WoA) was helpful for providing an overall idea on the best and the worst carrier substrates for microalgal species attachment.

Change in *Stichococcus* sp. biofilm physical properties was measured over total period of 35 days. Continuous measurement of biofilm physical properties revealed that EPS secretion influenced the physical properties of the biofilm. Self-induced breakage of EPS at the end of the exponential growth cycle changed the biofilm polarity. Biofilm polarity decreased with EPS concentration, inhibiting recruitment of cells and promoting natural detachment.

Monod model designed for planktonic growth was tested in growth evaluation of nutrient-dependent biofilm. Monod model was chosen due to its simplicity and low data input in comparison to models designed for description of biofilm growth kinetics. The equation matched the experimental data for initial biofilm development; therefore it could be successfully applied in estimation of growth in cyclic biofilm-based reactors.

6.2 Introduction

Non-suspended systems for microalgal cultivation are a promising alternative to traditional methods of growth [50]. Biofilm bioreactors are characterised by lower water consumption [16], higher biomass concentrations [7] [65], higher biomass productivity [17] and lower costs of production. Appropriate substrate selection is crucial for biofilm-based systems design, as material properties contribute to the overall system performance. Prize and durability of selected material are also very important; cheap and sturdy substrates decrease scale-

up costs. Testing is time and cost consuming, but attachment prediction tools can help in substrate selection without the need for expensive experimental trials. Unfortunately, studies that test prediction tools' applicability and agreement with empirical data are very limited, especially for microalgal biofilms. In this chapter, different modelling theories will be used to predict biofilm deposition and growth. Predictions will be compared to empirical data.

6.2.1 Cell attachment on surfaces

The initial step of biofilm development, cell attachment, is a complex process of colonisation of microbes on surfaces which involves many chemical and physical interactions [341]. The process is controlled by different biological, such as production of extracellular polymeric substances and cell-bound polymers, and environmental parameters, such as temperature, pH and nutrients availability [312].

Before colonisation, the substrate needs to be covered with a thin layer of conditioning film [342]. This layer of inorganic and organic compounds (e.g. proteins and/or polysaccharides) present in the aqueous matrix is usually formed within seconds after immersion [341]. The conditioning film has an influence on the physico-chemical properties of the carrier substrate and changes its surface charge, potential and tension to promote microbial attachment. Moreover, the shear resulting from liquid flow is reduced in the hydrodynamic boundary layer, closed to the conditioning film, providing a turbulence-free environment for microbial cells to attach. In practice, a conditioning film is created on any substrate and microorganisms are not involved in its formation.

Physical forces such as diffusion, convection or active movements or sedimentation lead microalgal cells from the bulk liquid towards the carrier substrate [343]. Both repulsive and attractive forces govern primary attachment of microbes [344]. They act when a cell is at 10-20 nm distance from the surface: Van der Waals and acid-base interactions are attractive forces acting on the cells to promote adhesion [345], whilst electrostatic interactions are repulsive forces resulting from the overlapping of substratum-cell electrical

double layer [222]. When a negatively charged cell overcomes the repulsive interactions, a reversible attachment can occur. Immobilised cells will create micro-colonies [346] and start releasing extracellular polymeric substances (EPS) that help in the formation of the biofilm community. Biofilms will then enter exponential growth phase, which is observed as a rapid increase in population. At this point, mature biofilm starts to be mostly governed by biological processes [312], and the impact of physical and chemical interactions declines.

6.2.2 Biofilm growth

Reversibly attached cells will start releasing polymeric substances that will entrap them into the biofilm matrix and create a well-organised, complex community of irreversibly attached microalgal cells. Various processes take place inside mature biofilms. Cell multiplication, release of EPS, transport of nutrients, production of DNA and extracellular products [347] are just examples of the activities performed by the biofilm community. Further growth of the biofilm is promoted by cell division and co-aggregation. At this stage, cell-substrate repulsive forces and van der Waals forces do not have any significant impact on biofilm-embedded cells [341]. The whole biofilm acts as a complex organism. Although matrix-embedded cells do not move and only few cells can communicate between each other, cellular interactions still play an essential role in the community [15] and influence its health [348].

Division, growth and death of cells are processes that take place simultaneously during biofilm development. When the rate of cells division is the same as rate of cells death, biofilm reached stationary growth phase.

The biofilm growth cycle ends with the death phase followed by sloughing. When the community runs out of nutrients, cells begin to die. It is observed as a natural phenomenon of detachment, where big clusters of microorganisms fall off the biofilm. The detachment is often triggered by the microorganisms themselves. Microbial community at the end of the growth cycle start releasing enzymes that are responsible for breaking down the EPS matrix [332] and trigger sloughing. The aim of auto-detachment is to preserve alive cells from

dying. EPS-free cells are able to move away from the nutrient deficient biofilm community and adhere to a new place richer in nutrients.

6.2.3 Biofilm formation models

Knowledge on attachment mechanisms would give an insight into the factors that may inhibit or enhance microbial adhesion to substrates. The primary colonisation of microorganisms is mostly governed by physical and chemical interactions that take place between cell, liquid medium and substrate. Literature reports theoretical studies on microbial adhesion in terms of work of attachment, however most of them are characterised by certain limitations and are not always able to quantitatively predict cell adhesion. There is still a visible lack of understanding of quantitative relation between theoretical attachment and experimental data; mechanism of microalgal attachment is not well investigated [187]. Table 12 summarises some of the studies on four of the main investigated attachment theories/models: Derjaguin-Landau-Verwey-Overbeek (DLVO), extended Derjaguin-Landau-Verwey-Overbeek (XDLVO), van Oss-Chaudbury-Good (VCG) and thermodynamic (TD). Unfortunately, in most cases the models failed to predict the adhesion. Prediction tools are important from an engineering point of view, as they significantly shorten the time required to perform experimental testing. Experimental investigations on the applicability of material as attachment substrate would require inoculation tests on many different materials, which would take time and money.

Ozkan and Berberoglu [186], Cui and Yuan [218], Prochazkova *et al.* [349] and Klein *et al.* [173] in their studies on theoretical adhesion, claimed that most of the work done on bacterial attachment can be applied to microalgal systems.

Table 12 Adhesion theories in relation to experimental data

Reference	Number of species	Microorganisms types	Number of materials	Material types	Outcome
Thwala *et al.*, 2013 [345]	2	*Pseudomonas putida, Bacillus subtilis* (bacteria)	5	membranes	XDLVO theory predicted that attachment would take place, but the value of adhesion do not relate to quantitative attachment
Feng *et al.*, 2015 [350]	4	*Escherichia coli, Listeria monocytogenes, Staphylococcus aureus, Staphylococcus epidermidis* (bacteria)	1	alumina	XDLVO is not a good quantitative prediction of bacterial attachment
Nguyen *et al.*, 2011 [351]	6	*Campylobacter jejuni* (C939a, C977a, ATCC 33560), *Salmonella* (Sofia, Infantis, Typhiurium) (bacteria)	1	stainless steel 304	XDLVO theory is not able to predict quantitative attachment- it predicted attachment of three strains, but failed to predict attachment of 3 other.
Ozkan and Berberoglu, 2013 [157]	10	*Ankistrodesmus falcatus var. stipitatus , Botryococcus braunii, Botryococcus sudeticus, Chlorella Vulgaris, Nannochloris oculata, Scenedesmus dimorphus, Nannochloris* sp. (microalgae), *Amphora coffeaeformis, Cylindrotheca fusiformis, Nitzschia frustulum* (diatoms)	6	glass, ITO, stainless steel, polybarbonate, polyethylene, polystyrene	XDLVO equation can be used to estimate adhesion strength
Ozkan and Berberoglu, 2013 [186]	2	*Chlorella vulgaris, Botryococcus sudeticus* (microalgae)	2	glass, indium tin oxide	TD model successfully predicted the adhesion of *C. vulgaris* to glass and ITO and attachment of *B. sudeticus* to ITO. XDLVO equation was the best

					among all other adhesion approaches (DLVO and thermodynamic) in prediction of adhesion strength and attachment.
Prochazkova *et al.*, 2013 [349]	1	*Chlorella vulgaris* (microalgae)	2	magnetic beads (diethylaminoethyl and polyethyleneimine functional groups)	XDLVO was not able to capture additional interactions between polyetyleneimide and *C. vulgaris* surface
Cui and Yuan, 2013 [218]	2	*Scenedesmus dimorphus, Nannochloropsis oculata* (microalgae)	5	nylon, polycarbonate, polypropylene, glass	TD model qualitatively matched experimental data for the same type of material (plastics) but did not predict the attachment on various materials
Ista and Lopez, 2013 [352]	1	*Cobetia marina* (bacteria)	1	oligo(ethylene-glycol) monolayers	VCG model did not relate to attachment profiles
Fletcher and Pringle, 1985 [174]	8	*Bacillus filicolonicus, Bacillus pacificus, Micrococcus* sp., *Flavobacterium uliginosum, Pseudomonas* sp., *Corynebacterium erythrogenes, Vibrio fisheri* (bacteria)	1	polystyrene	The attachment increases with values of work of attachment (from 75 to 105 mJ/m^2) and after reaching peak, it decreases.

6.2.3.1 DLVO and XDVLO models

The DLVO model of attachment assumes that cells behave as spherical inert particles [222]. The method was developed independently by Derjaguin and Landau in 1941 [353], and by Verwey and Overbeek in 1948 [354]. Extended DLVO (XDLVO) is an extension of the original DLVO method and takes into account additional relations, such as acid-base interactions, that were not included in the basic DLVO [186].

The XDLVO equation showed the best correlation with the experimental data of a freshwater microalgae attachment in a study by Ozkan and Berberoglu [186]. Several of the assumptions made in the work could undermine the results. Of the four points used for each material one was 'a starting point' (0,0) and no error was displayed, so it was not clear how many measurements were taken. Another work of Ozkan and Berberoglu [217] tested the XDLVO model on a broader range of strains and materials. Authors stated that the model could be used for predicting strength of adhesion. However, the work was purely theoretical, with no experimental investigation.

The non-covalent nature of XDLVO results in its lower applicability as prediction tool in microbial attachment where covalent bonds are involved. In 2013, Prochazkova *et al.* [349] demonstrated that the XDLVO model is not applicable when additional interactions, in this case covalent bonding, take place between algae and polyethylenimide. The outcome of the work was that the equations describing adhesion are not good for predicting quantitative attachment, but rather qualitative [345] [350] [351].

6.2.3.2 VCG model

VCG is a colloidal model for the predictions of microbial attachment by van Oss, Chaudbury and Good [355]. The VCG model uses Lifshitz-van der Waals, Lewis acid-base and electrostatic interactions [356]. The model can help the evaluation of surface and interfacial tensions [357], although it did not successfully predict *Cobetia marina* attachment profiles in the study performed by Ista and Lopez [352].

6.2.3.3 TD model

The thermodynamic model describes the decrease in free energy of the system that results from spontaneous changes. The method main limitation is that the system is assumed to be at equilibrium state [358]. There are many mechanisms that have not been accounted for in the thermodynamic model, for example polymer or acid-base interactions. Despite the drawbacks, the thermodynamic model was proved successful in predictions in adhesion of some microbial species, as showed by Cui and Yuan [218] for *S. dimorphus* or *N. oculata*.

6.2.3.4 Work of attachment- TD model principles

Cui and Yuan [218] constructed a thermodynamic equation of cell-substrate attachment, which was validated by experiments on *Scenedesmus dimorphus* and *Nannochloropsis oculata* microalgae. According to their equation, work of attachment consists of three interfacial free energies (Equation 9). Those are the energies that are arising between cell and liquid, substratum and liquid, and cell and substratum. The sum of interfacial free energy between the cell and the liquid, and interfacial free energy between substratum and the liquid lessen by the interfacial free energy between cell and substratum, gives the work of attachment/adhesion (Equation 9).

$$\Delta G_{adh} = \gamma_{cl} + \gamma_{sl} - \gamma_{cs}$$

Equation 9 Work of adhesion/attachment

Using Owens and Wendt theory and Good's Equation, Cui and Yuan developed following formulas for the interfacial free energies (Equation 10, Equation 11, Equation 12):

$$\gamma_{cs} = \gamma_c + \gamma_s - 2\sqrt{(\gamma_c^d \gamma_s^d)} - 2\sqrt{(\gamma_c^p \gamma_s^p)}$$

Equation 10 Cell-substratum interfacial free energy

$$\gamma_{cl} = \gamma_c + \gamma_l - 2\sqrt{(\gamma_c^d \gamma_l^d)} - 2\sqrt{(\gamma_c^p \gamma_l^p)}$$

Equation 11 Cell-liquid interfacial free energy

$$\gamma_{sl} = \gamma_s + \gamma_l - 2\sqrt{(\gamma_s^d \gamma_l^d)} - 2\sqrt{(\gamma_s^p \gamma_l^p)}$$

Equation 12 Substratum-liquid interfacial free energy

Where:

c- cell

s- solid/substrate

l- liquid

After introducing two components of surface free energy (dispersive energy and polar energy), the surface free energies become:

$$\gamma_s = \gamma_s^d + \gamma_s^p$$

Equation 13 Substrate surface free energy

$$\gamma_c = \gamma_c^d + \gamma_c^p$$

Equation 14 Cell surface free energy

$$\gamma_l = \gamma_l^d + \gamma_l^p$$

Equation 15 Liquid surface free energy

Inserting them into the equations Equation 10, Equation 11 and it gives:

$$\gamma_{cs} = \gamma_c^d + \gamma_c^p + \gamma_s^d + \gamma_s^p - 2\sqrt{(\gamma_c^d \gamma_s^d)} - 2\sqrt{(\gamma_c^p \gamma_s^p)}$$

Equation 16 Cell-substratum interfacial free energy

$$\gamma_{cl} = \gamma_c^d + \gamma_c^p + \gamma_l^d + \gamma_l^p - 2\sqrt{(\gamma_c^d \gamma_l^d)} - 2\sqrt{(\gamma_c^p \gamma_l^p)}$$

Equation 17 Cell-liquid interfacial free energy

$$\gamma_{sl} = \gamma_s^d + \gamma_s^p + \gamma_l^d + \gamma_l^p - 2\sqrt{(\gamma_s^d \gamma_l^d)} - 2\sqrt{(\gamma_s^p \gamma_l^p)}$$

Equation 18 Substratum-liquid interfacial free energy

Cui and Yuan derived the following equation for the work of attachment/adhesion (Equation 19):

$$\Delta G_{adhesion} = \gamma_c^d + \gamma_c^p + \gamma_l^d + \gamma_l^p - 2\sqrt{(\gamma_c^d \gamma_l^d)} - 2\sqrt{(\gamma_c^p \gamma_l^p)} + \gamma_s^d + \gamma_s^p + \gamma_l^d + \gamma_l^p - 2\sqrt{(\gamma_s^d \gamma_l^d)} - 2\sqrt{(\gamma_s^p \gamma_l^p)} - \gamma_c^d - \gamma_c^p - \gamma_s^d - \gamma_s^p + 2\sqrt{(\gamma_c^d \gamma_s^d)} + 2\sqrt{(\gamma_c^p \gamma_s^p)}$$

Equation 19 Work of attachment/adhesion

Equation 19 takes the following form after rearrangement:

$$\Delta G_{adhesion} = 2\gamma_l^d + 2\gamma_l^p - 2\sqrt{(\gamma_c^d \gamma_l^d)} - 2\sqrt{(\gamma_c^p \gamma_l^p)} - 2\sqrt{(\gamma_s^d \gamma_l^d)} - 2\sqrt{(\gamma_s^p \gamma_l^p)} + 2\sqrt{(\gamma_c^d \gamma_s^d)} + 2\sqrt{(\gamma_c^p \gamma_s^p)}$$

Equation 20 Work of attachment/adhesion simplified

6.2.4 Planktonic growth prediction models

6.2.4.1 Monod kinetics for nutrient- dependent planktonic growth

Monod kinetic was the first model used for evaluating the growth rates of suspended microorganisms in nutrient-dependent aquatic environments [58]. The model is a simplified version of the phenomena that occur empirically [359].

$$\mu = \mu_{max} \frac{S}{K_S + S}$$

Equation 21 Monod equation

where

μ – specific growth rate

μ_{max}- maximum growth rate

K_S- growth affinity constant

S- concentration of growth-driving nutrient

Monod equation has many limitations [360] [361], although it is less demanding than other complicated growth prediction models. The equation is very simple whereas the growth of microorganisms in relation to nutrient concentration is a very complex function [359]. Growth affinity constant introduced by Monod is influenced by bacterial adaptations. Microorganisms are able to adjust to varying environment; therefore it is very hard to describe the growth of cells using simple equations.

6.2.4.2 Population Balance Equations (PBEs) models

Other mathematical models have been developed in order to predict the growth of microbial community in planktonic state [362] [363] [364] [365], some of which partially rely on Monod kinetics [145] [361] [366] [367] [368] [369] [370] [371]. Population balance equations (PBEs) are widely used to describe cell growth and division. PBEs are set of partial differential equations that base on internal coordinates of cells [372]. They describe the growth based on cell size, age, morphology or mass. Their main advantage over other existing mathematical models is that they treat cell growth and division separately, which better reflects the actual growth phenomenon [373]. However, the main disadvantage of PBEs over traditional, simple Monod model is that they require large data input and are very complex and time consuming. Models proposed by Rading *et al.* [374] and Concas *et al.* [145] take into account multiple cell division. Rading *et al.* did not account for the influence of light and nutrient availability. Model proposed by Concas *et al.* utilised Monod-type dependence for the description of the mass rate in relation to nutrients concentration. In their model, cells are able to produce multiple numbers of daughter cells, up to eight (octonary fission). The results showed a high correlation between the model and experimental data for *Nannochloris eucaryotum* growth [145].

6.2.5 Sessile growth according to kinetic model

Growth of microbial cells in biofilms is more difficult to be modelled than growth in planktonic state. Nutrient consumption is not only limited by nutrient availability, but also by the access of cells to nutrients expressed by biofilm

thickness. Actual nutrient availability for whole biofilm community is corrected by introducing nutrient concentration profiles, as shown in Figure 39 [375].

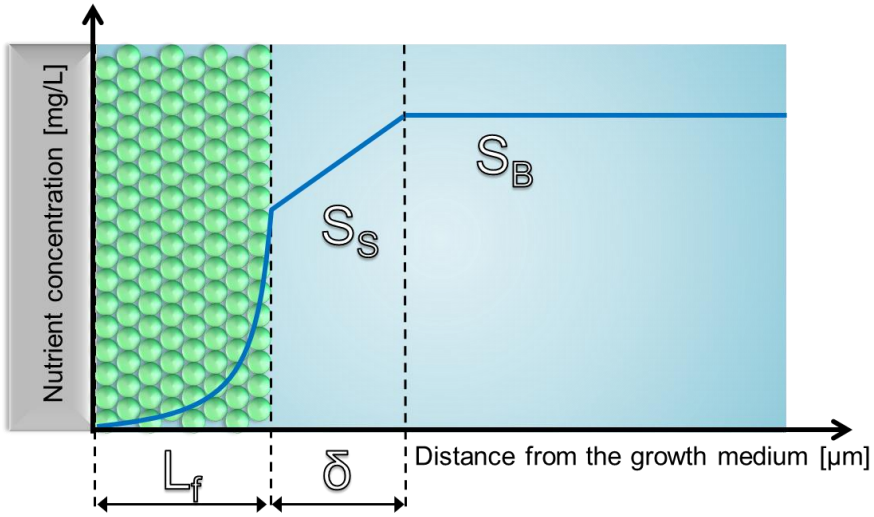


Figure 39 Nutrient concentration profile in homogenous biofilm

where

S nutrient concentration

S_B nutrient concentration at bulk solution

S_S nutrient concentration at the biofilm surface

z distance from the growth medium

L_f biofilm thickness

δ mass transfer boundary layer thickness

— nutrient availability

Evaluation of mass transfer by diffusion is essential to accurately predict biofilm development, as deeper layers of biofilm would not receive the same amount of nutrients as the cells on biofilm-liquid interface.

6.2.5.1 Monod kinetics for sessile growth

Complicated mechanism of nutrient utilisation by biofilms requires more advanced Monod kinetics. Therefore, two profiles of nutrient availability need to be evaluated: external and internal to biofilm. External profile is expressed by nutrient flux (F) as defined in Equation 22:

$$F = -\frac{D_W(S_B - S_S)}{\delta}$$

Equation 22 Nutrient flux

where:

F- flux of the nutrients to the biofilm

D_W- diffusivity of nutrients in water

Internal profile is expressed with nutrient transportation by diffusion and microbial consumption of nutrients. Expressing the rate of mass transfer by diffusion with Fick's law and microbial consumption rate with Monod-type equation, following relationship takes place internally at steady-state [375]:

$$D_f \frac{d^2S}{dz^2} = \mu_{max} \frac{S}{K_S + S} \cdot \frac{\rho}{Y_{x/s}}$$

Equation 23 Internal biofilm nutrient availability

where:

D_f- average effective diffusivity [m^2/s]

ρ- average biofilm density [kg/m^3]

$Y_{x/s}$- yield coefficient [kg of algae/kg of nutrients]

The presented model is a description of biofilm growth kinetics rather than an effective tool for quantitative growth predictions. Large data input, including structural characteristics of biofilm such as biofilm thickness, boundary layer thickness, yield coefficient and mass transfer through biofilm make the model complicated and time consuming. In order to describe biofilm growth with the model, continuous measurements on biofilm parameters are required. Moreover, the model does not take into account complex biofilm mechanics, such as hydrodynamics, varying density across biofilm and effective diffusivity

gradients. To date, there is no accurate model for biofilm growth predictions [375].

6.2.5.2 3D structural models

Some mathematical models are used for predicting 3D structure of biofilm; channels, flow pattern or cell clusters [368]. The main purpose of such models is to obtain a valid output structure formation and to describe growth of biofilm [376]. Multidimensional biofilm modelling is characterised by high accuracy. However, it is quite problematic from process engineering point of view, as it requires a large amount of input data.

6.2.6 Biofilm deposition and growth models- research needs

Studies on microalgal primary attachment predictions are very limited. Small number of materials and strains were tested, so it was not possible to fully assess the potential of adhesion prediction tools. Hence in this chapter, the thermodynamic model was chosen for the theoretical evaluation of the work of attachment of 4 microalgal species, because it is the simplest among discussed adhesion prediction tools: it is not time-consuming, and requires low data input. Primary colonisation on nine different materials (steels, plastics and glass) was confronted with the thermodynamic equation. Work of attachment was linked to the experimental data with the help of Pearson correlation coefficient.

Predicting further biofilm development is also important. The main purpose of mathematical modelling is to predict growth dynamics in bioreactors, mostly for bacteria and mammalian cells [377]. Not many PBEs' mathematical simulations have been investigated for microalgae [145] and theoretical models that describe microalgal biofilm growth kinetics are difficult to apply and require a lot of input data. However, in biofilm bioreactors, only initial development of biofilm is essential from reactor design point of view. Further biofilm maturation does not have to be predicted, as in systems with re-growth cycle biofilm is not allowed to mature. Applicability of the simplest planktonic growth prediction model in empirical initial biofilm growth estimations was investigated. Monod

model was used to evaluate *Stichococcus* sp. and *C. saccharophila* nutrient-dependent biofilm growth.

6.3 Materials and methods

6.3.1 Microalgal strains and growth conditions

Aeroterrestrial strains *Stichococcus* sp. CCAP 379/30 and *C. luteoviridis* CCAP 211/3 and freshwater strains *Haematococcus pluvialis* CCAP 34/7 and *Chlorella saccharophila* CCAP 379/30 were obtained from Culture Collection of Algae and Protozoa, UK (CCAP).

Prior to inoculation, all cultures were cultivated in Bold Basal medium (BBM) in 250 ml Erlenmeyer flasks placed on orbital shaker Innova®44 at 180 RPM. Temperature inside the incubator shaker was maintained at 22°C. The continuous light intensity was equal to 42 µmol/m^2/s.

Adhesion materials were: stainless steel grade 316 bead blasted, stainless steel grade 316 sand polished, stainless steel grade 304 bead blasted, stainless steel grade 304 sand polished, polyvinyl chloride, glycol-modified polyethylene terephthalate, polypropylene, polystyrene and glass. All materials were cleaned with isopropanol prior to inoculation.

Inoculation, cell count, measurements of removal energies, SMP and EPS were performed using the same methods reported in Chapter 5.3. Cells were inoculated on materials by placing substrate and microalgal culture in polypropylene container. The container was sealed and cells were allowed to inoculate the materials overnight. Biofilm density was calculated by cell counting method with Improved Neubauer Haemocytometer (Weber England, depth 0.1mm, 1/400 mm^2) and optical density method carried out by a Jenway 6800 Double Beam UV/ViS Spectrophotometer. Removal energies were calculated by measuring the time and energy applied by Fisher Scientific FB 15051 ultrasonic bath at 37 kHz frequency to remove biofilm placed inside phosphate buffer. SMP and EPS concentrations were evaluated by measuring sugars by phenol-sulfuric acid assay [378] and proteins with Folin–Ciocalteu assay [293].

Experimental results obtained in Chapter 3 and Chapter 5 were used in this study for comparative purposes.

6.3.2 Determination of interface contact angle and free energy of adhesion of *Stichococcus* sp. biofilm

Biofilm-liquid interface contact angles (CA) were used in the calculation of surface free energies of growing *Sitchococcus* sp. biofilm on both polystyrene and polyethylene terephthalate. For that purpose, contact angles of three reference liquids were measured with Biolin Scientific Theta Lite Optical Tensiometer by sessile drop technique. Liquids with known surface tensions were used: water (γ_l^d=21.8 mJ/m^2, γ_l^p=51.0 mJ/m^2), diiodomethane (γ_l^d=50.9 mJ/m^2, γ_l^p= 0.0 mJ/m^2) and ethylene glycol (γ_l^d=32.8 mJ/m^2, γ_l^p=16.0 mJ/m^2). Prior to measurements, water contact angle was measured every 5 minutes in order to determine biofilm drying time [208]. When water contact angle became stable, contact angles with remaining reference liquids were tested. Contact angles were measured five times for each liquid, giving the total of 15 measurements per each sample. Each contact angle was recorded by OneAttention Software over the time period of 12 seconds. Surface free energies of biofilm were evaluated with OneAttension Software by OWRK/Fowkes method.

6.3.3 Total nitrogen and total phosphorous measurement

The total nitrogen (TN) and total phosphorous (TP) concentrations in BBM were measured every week for the total duration of the experiment, in order to evaluate availability of nutrients needed for Monod model (concentration of limiting nutrient) using Spectroquant® Cell Test Kits All samples were diluted prior to measurements, to ensure more accurate results.

Total N measurement was performed using Spectroquant® Nitrogen (total) Cell Test Kit 114537 0.5-15.0 mg/L (WVR, UK) following producer's instructions. Total nitrogen concentration was measured by Spectroquant®NOVA 60 photometer.

Total P was measured using Spectroquant® Phosphate Cell Test Kit 114543 0.05-5.0 mg/L (WVR, UK) following producer's instructions. The reaction time was 5 minutes, after which the sample was analysed by Spectroquant®NOVA 60 photometer.

6.3.4 Work of attachment evaluation

TD model predicts the attachment in terms of work of adhesion. According to Cui and Yuan [218], work of adhesion (ΔG_{adh}) is a work required to separate cell from substrate in liquid medium. It was calculated with Equation 24:

$$\Delta G_{adh} = 2\gamma_l^d + 2\gamma_l^p - 2\sqrt{(\gamma_c^d \gamma_l^d)} - 2\sqrt{(\gamma_c^p \gamma_l^p)} - 2\sqrt{(\gamma_s^d \gamma_l^d)} - 2\sqrt{(\gamma_s^p \gamma_l^p)} + 2\sqrt{(\gamma_c^d \gamma_s^d)} + 2\sqrt{(\gamma_c^p \gamma_s^p)}$$

Equation 24 Work of adhesion

Where:

ΔG_{adh}- work of adhesion

$\gamma_{l,c,s}^{d,p}$- dispersive/polar surface free energy of liquid/cell/substrate

The circularities of all microalgal strains exceeded 0.78, according to results from Chapter 3.4.3. Therefore, shapes of algal cells can be treated as spheres [198] and work of attachment can be calculated by thermodynamic equation.

6.3.5 Nutrient-dependent growth of biofilm evaluated by Monod equation

Nitrogen and phosphorous are nutrients that limit the growth of microalgal species [379]. Therefore, their concentration in BBM was monitored over the biofilm growth. The growth rate depends on either total nitrogen or total phosphorus concentrations in the medium, therefore both N- and P- dependent growth rates were calculated. Monod equation is given by Equation 25:

$$\boldsymbol{\mu = \mu_{max} \frac{S}{K_S + S}}$$

Equation 25 Monod equation

where

μ – specific growth rate

μ_{max}- maximum growth rate

K_S- growth affinity constant

S- concentration of limiting nutrient

In order to compare number of cells predicted by Monod equation with experimental data, the growth rate was used in projection of *Stichococcus* sp. and *C. saccharophila* growth in relation to available nutrients (Equation 26).

$$\mu = \frac{\ln(\frac{\rho}{\rho_0})}{(t - t_0)}$$

Equation 26 Specific growth rate

where

ρ – number of cells attached onto substrate [cells/cm^2] at time=t [days]

ρ_0- number of cells attached onto substrate [cells/cm^2] at initial time=t_0 [days]

Initial attachment of *Stichococcus* sp. and *C. saccharophila* was used in the projection of further growth on PETG and PS substrates, predicted by Monod kinetics (Equation 27).

$$\rho = \rho_0 \cdot \exp(\mu \cdot (t - t_0))$$

Equation 27 Initial attachment

The number of attached cells according to Monod equation was calculated for each day and compared with experimental data. In order to illustrate the method for growth evaluation, the example calculations are showed below:

$$\rho_4 = \rho_1 \cdot \exp(\mu_4 \cdot (t_4 - t_1))$$

Equation 28 Attachment at day 4

$$\rho_7 = \rho_4 \cdot \exp(\mu_7 \cdot (t_7 - t_4))$$

Equation 29 Attachment at day 7

where

ρ_1- number of cells attached onto substrate [cells/cm^2] at initial time=t_1=1 [days] evaluated experimentally

ρ_4- number of cells attached onto substrate [cells/cm^2] at time=t_4=4 [days] evaluated by Monod equation

ρ_7- number of cells attached onto substrate [cells/cm^2] at time=t_7=7 [days] evaluated by Monod equation

μ_4- specific growth rate [day^{-1}] at time=t_4=4 [days] evaluated by Monod equation

μ_7- specific growth rate [day^{-1}] at time=t_7=7 [days] evaluated by Monod equation

6.3.6 Pearson correlation coefficient

The theoretical work of attachment and experimental adhesion of cells to substrate were correlated using Pearson coefficient [380]. The coefficient describes the strength of linear association between two data sets and is represented by Equation 30. Higher absolute value of the coefficient means higher linear association between two data sets.

$$P_c = \frac{\sum_i (x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_i (x_i - \bar{x})^2 \sum_i (y_i - \bar{y})^2}}$$

Equation 30 Pearson correlation coefficient

where, in this Chapter:

P_c- Pearson correlation coefficient

$\bar{x}$ – average value of work of attachment

$\bar{y}$ – average value of microalgae adhesion

x_i- work of attachment for certain microalgae-material pairing

y_i- adhesion of certain microalgae-material pairing

Correlation is supported by two-tailed t-test in order to extract the significance of the linear association [381]. T-value has been calculated using Equation 31:

$$t = \frac{P_c}{\sqrt{\frac{1 - {P_c}^2}{d_f}}}$$

Equation 31 Two-tailed t-test

$$d_f = N - 2$$

Equation 32 Degrees of freedom

where:

t- two-tailed t-test value

d_f- degrees of freedom

N- number of variables (tested materials)

The correlation is significant at a given level if calculated t-value is equal or greater than the critical t-value specified for the same level of significance and degree of freedom [382]. Significance level can be defined as a probability of rejection of the stated hypothesis. Therefore, if a significance level α is equal to 0.2 it means that the probability that the two data sets considered are correlated is equal to 0.8.

6.4 Results and discussion

6.4.1 Microalgal primary colonisation and work of attachment

The work of attachment of *Stichococcus* sp., *C. luteoviridis*, *C. saccharophila* and *H. pluvialis* for 9 materials in relation to the experimental primary colonisation is presented in Figure 40.

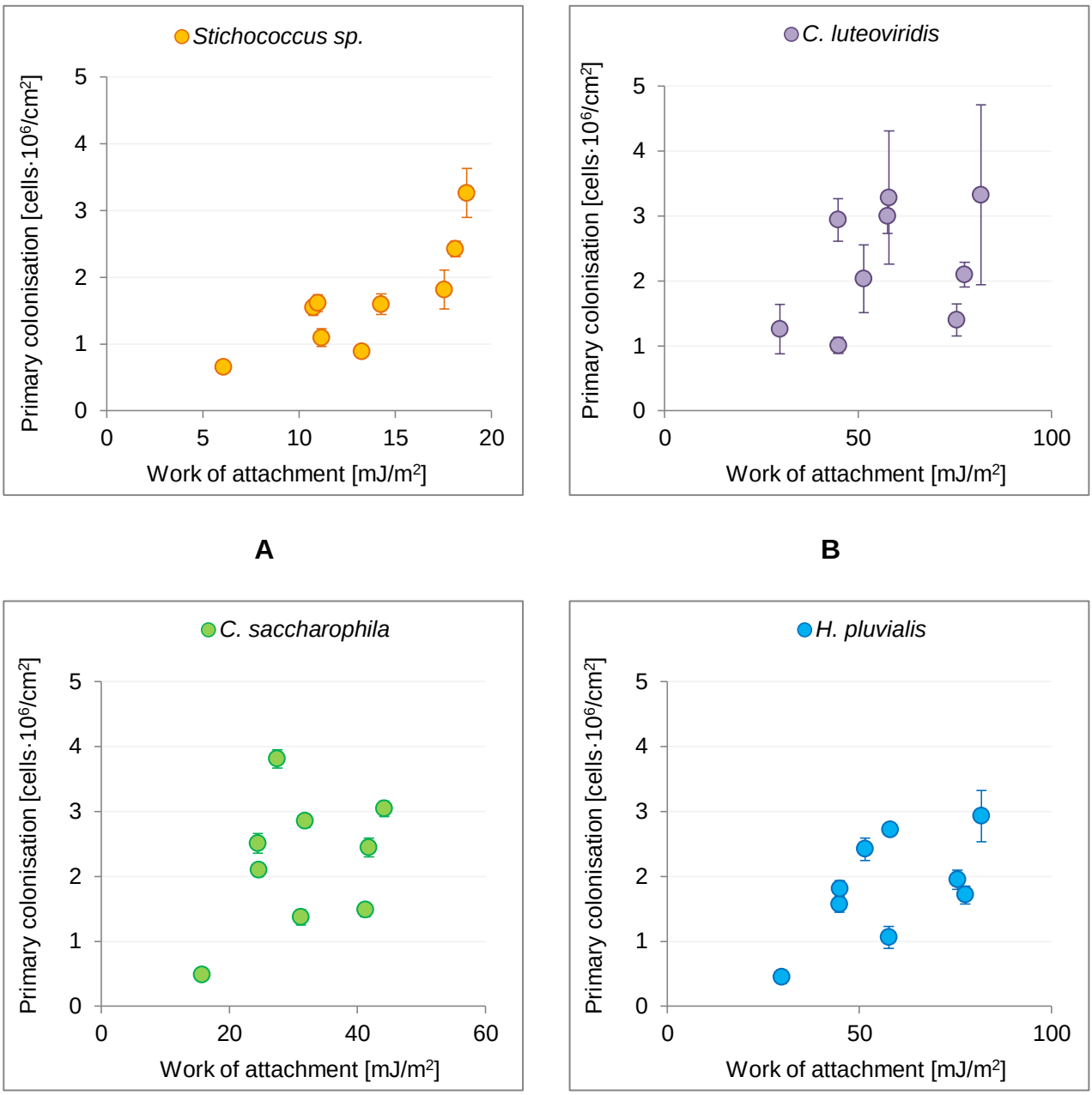


Figure 40 Work of attachment and primary colonisation of microalgae

Attachment predictions for *Stichococcus* sp. indicated that the highest energy of adhesion was obtained for PS, as shown in Figure 40 A. The energy required to remove *Stichococcus* sp. cell from this substrate was equal to 18.7±1.1 mJ/m^2. Glass exhibited the lowest work of attachment among all tested substrates: only 6.1±0.6 mJ/m^2 was needed in order to detach *Stichococcus* sp. cells.

Stichococcus sp. on stainless steels 316 BB and 304 SP had almost the same energy of adhesion: 14.3±1.2 and 13.3±0.9 mJ/m^2, respectively. This is especially interesting as both substrates differ in chemical composition and structure. Bead blasted steel is a rough material with an average surface roughness equal to 6.69±0.29µm, while sand polished steel is a substrate with smooth finish and R_a = 0.43±0.01µm. Moreover, 316 BB contains antifouling molybdenum; whereas 304 graded SP steel did not. Nevertheless, both steels share the same value of polar component of surface free energy. It is almost two times lower than the polar part of remaining two steel materials: 7.03 and 6.90 mJ/m^2 (304 SP and 316 BB) compared to 10.35 and 12.86 mJ/m^2 (316 SP and 304 BB). As mentioned in Chapter 3.4.8.2, polarities of materials were not correlated to experimental primary attachment of *C. saccharophila, C. luteoviridis, Stichococcus* sp. and *H. pluvialis*. However, due to the constant SFE of *Stichococcus* sp. and liquid medium, dispersity and polarity of material are the only components that affect WoA in this study.

In general, *Stichococcus* sp. on plastic materials showed higher WoA than on steel materials, 17.5-18.7 mJ/m^2 in comparison to 10.7-14.3 mJ/m^2. The only exception was polypropylene that exhibited very small WoA equal to 11.0±1.1 mJ/m^2. Similarly as in the case of steel materials, lower WoA on PP could be explained by very high polarity of this material. SFE polar component of PP was equal to 12.4 mJ/m^2, which was 10 times higher than polarity of other plastics. PS, PETG and PVS were highly hydrophobic, therefore the adhesion of *Stichococcus* sp. was favoured on those materials.

The theoretical data on work of attachment reflects the quantitative attachment of *Stichococcus* sp. The lowest WoA on glass and highest on PS was confirmed by empirical attachment. Adhesion on steel materials was lower than adhesion on plastics; 0.9-1.6cells·10^6/cm^2 in comparison to 1.6-3.3cells·10^6/cm^2 for steels and plastics, respectively. Moreover, the TD model predicted that polypropylene will be the least effective material among plastics.

WoA calculated for aeroterrestrial *C. luteoviridis* is presented in Figure 40 B. The correlation between WoA and attachment was not as satisfactory as in the

case of *Stichococcus* sp. The lowest energy of adhesion by *C. luteoviridis* was obtained on glass, although the actual attachment was the worst on PP. Microalgae adhered in higher numbers to steel materials than plastics (with the exception of PS), which was not predicted by the TD model. Attachment on PVC, PETG and PP was forecasted to be two times lower than the actual number of cells that deposited on those materials. It was the highest disproportion between theoretical and empirical results for *C. luteoviridis* attachment. It is possible that attractive forces acting between those plastics and *C. luteoviridis* cell were greater than those predicted by TD model. Acid-base interactions are not accounted in thermodynamic work of attachment. The interfacial free energy resulting from acid-base interactions is strongly dependent upon type of plastic and type of wetting agent [383]. As wetting agent in all experiments performed in this chapter was BBM, the value of acid-base interactions will depend on type of plastics. The trend of higher than expected attachment was observed for PVC and PETG for all microalgal strains.

Similarly to *C. luteoviridis*, freshwater *C. saccharophila* primary attachment was not well predicted by the thermodynamic model (Figure 40 C). Work of attachment calculations suggested that microalgae will attach in highest numbers to PS. Notwithstanding high adhesion values for PS, the best primary colonisation took place on sand polished stainless steel 316 grade. *C. saccharophila* cells did not adhere well to glass, which also had the lowest work of attachment of all the materials considered.

The work of attachment did not quantitatively match the experimental data for *H. pluvialis*, although it was able to show the likelihood of attachment (Figure 40 D). The highest and the lowest WoA matched experimental attachment, with 29.8 mJ/m^2 and 4.5±1 cells·10^5/cm^2 for glass and 81.8 mJ/m^2 and 2.9±0.4 cells·10^6/cm^2 for PS. The thermodynamic model suggested that *H. pluvialis* adhesion on PP would be around two times lower than on PS, which was confirmed by experimental data. However, the thermodynamic model failed to predict that adhesion on remaining plastics would also be much lower than on

PS. According to the model, adhesion on all steel should be lower than on plastics with very similar energy of adhesion equal from 44.8 mJ/m^2 for bead blasted grade 304 to 58.0 8 mJ/m^2 for bead blasted 316. In reality, *H. pluvialis* colonised very well both bead blasted and sand polished stainless steels of grade 316, but the attachment on grade 304 was significantly lower especially on sand polished steel; 2.7±0.1 and 2.4±0.2 cells·10^6/cm^2 compared to 1.6±0.1 and 1.1±0.2 cells·10^6/cm^2, respectively.

So, the thermodynamic model successfully predicted quantitative adhesion of *Stichococcus* sp. cells, although it did not matched experimental attachment data for remaining strains.

6.4.1.1 Pearson correlation coefficient

The Pearson coefficient for the work of attachment and the experimental adhesion of *Stichococcus* sp. was equal to 0.8166 with two-tailed t-test passed at the significance level 0.01, which indicated high correlation. High correlation implicates that the higher *Stichococcus* sp. microalgae specie attachment characteristics, the higher WOA independently of the material used.

WoA of both *Chlorella* strains did not match experimental data, with very low Pearson correlation coefficient equal to 0.3473 for *C. luteoviridis* and 0.3407 for *C. saccharophila*. Two-tailed t-test was not passed, indicating that the relation between empirical and theoretical data was not statistically significant. However, the test was passed for *H. pluvialis* attachment, meaning that correlation held true with significance level equal to 0.1 implying positive linear correlation. T-test passed at the significance level of 0.1 means that there is 90% probability that the correlation holds for the data. Nevertheless, the Pearson coefficient was equal to 0.5939, indicating weak positive linear correlation.

Pearson correlation coefficient was used to evaluate the relationship between material dispersity and polarity. There was no connection between *Stichococcus* sp. empirical data and the dispersive component of surface free energy of the tested materials, but there was a relationship with polarity of

substrate, with Pearson coefficient equal to -0.7337, which has passed the t-test at significance 0.05, indicating negative linear correlation. The adhesion to materials with higher polarity will be lower than to materials with low polarity for *Stichococcus* sp.

In this study, the polarity of substrates is strictly dependent upon water contact angle with strong negative linear correlation equal to -0.9557 and passed the two-tailed t-test with the significance level of 0.001. It can be stated that hydrophilicity of material also indicated its polarity. As it was showed by Cooksey and Wigglesworth-Cooksey [155] and Klein *et al.* [173], hydrophobic substrates were favourable materials for microbial attachment. The same relation was observed in the case of *Stichococcus* sp. and 9 tested materials. The adhesion was decreasing with increasing hydrophilicity of substrates.

Cui *et al.* [35] suggested that SFE of materials is an important factor, influencing the adhesion more than substrate roughness. Fletcher and Pringle [174] stated that adhesion decreases with increasing SFE of material. Nevertheless, in this chapter there was no visible relation between SFE of materials and their empirical attachment.

To conclude, it is clear that the work of attachment evaluated using the thermodynamic model was strongly correlated to the experimental data of adhesion for *Stichococcus* sp. In addition, WoA was more accurate in predicting the attachment of *Stichococcus* sp. than the values of polarity of the different materials

6.4.1.2 Stichococcus sp. *mechanism of attachment*

Successful implementation of the thermodynamic equation in predictions of *Stichococcus* sp. biofilm deposition allowed the identification of physical interactions that governed the attachment of this microalga. The main factors taken into account in the thermodynamic model were: (1) surface free energies of the substrate, (2) cell and (3) medium. As SFE is expressed by both polar and dispersive components, their importance in the attachment process of *Stichococcus* sp. was very high. Electrostatic forces acted repulsively on

Stichococcus sp. cell, which was indicated by higher Coulomb forces [221] expressed by polarity of cell (γ^{p}_{cell}=30.024 mJ/m^2). The SFE polar component described the strength of the repulsive interactions, whereas the dispersive component was connected to attractive forces. It is evident that attractive van der Waals and repulsive electrostatic interactions are the main processes that take place at cell-substratum interface during *Stichococcus* sp. attachment. It was presumably the lack of polyols that did not alter the adhesion of *Stichococcus* sp. Polyols are compounds that help against environmental stresses and are often observed in aeroterrestrial microalgae [250]. The production of polyols would contribute to biological interactions, not accounted in the thermodynamic model. However, *Stichococcus* sp. was proven to not secrete effective stress metabolites, therefore there were no additional interactions involved in its adhesion.

6.4.2 Work of attachment in relation to biofilm development andremoval and EPS excretion

6.4.2.1 WoA in relation to EPS secretion

The work of attachment values calculated over the 35 days of *Stichococcus* sp. biofilm cultivation are summarised in Table 13. Results at 0th day show the work of attachment calculated for non EPS-bound cells, before biofilm formation.

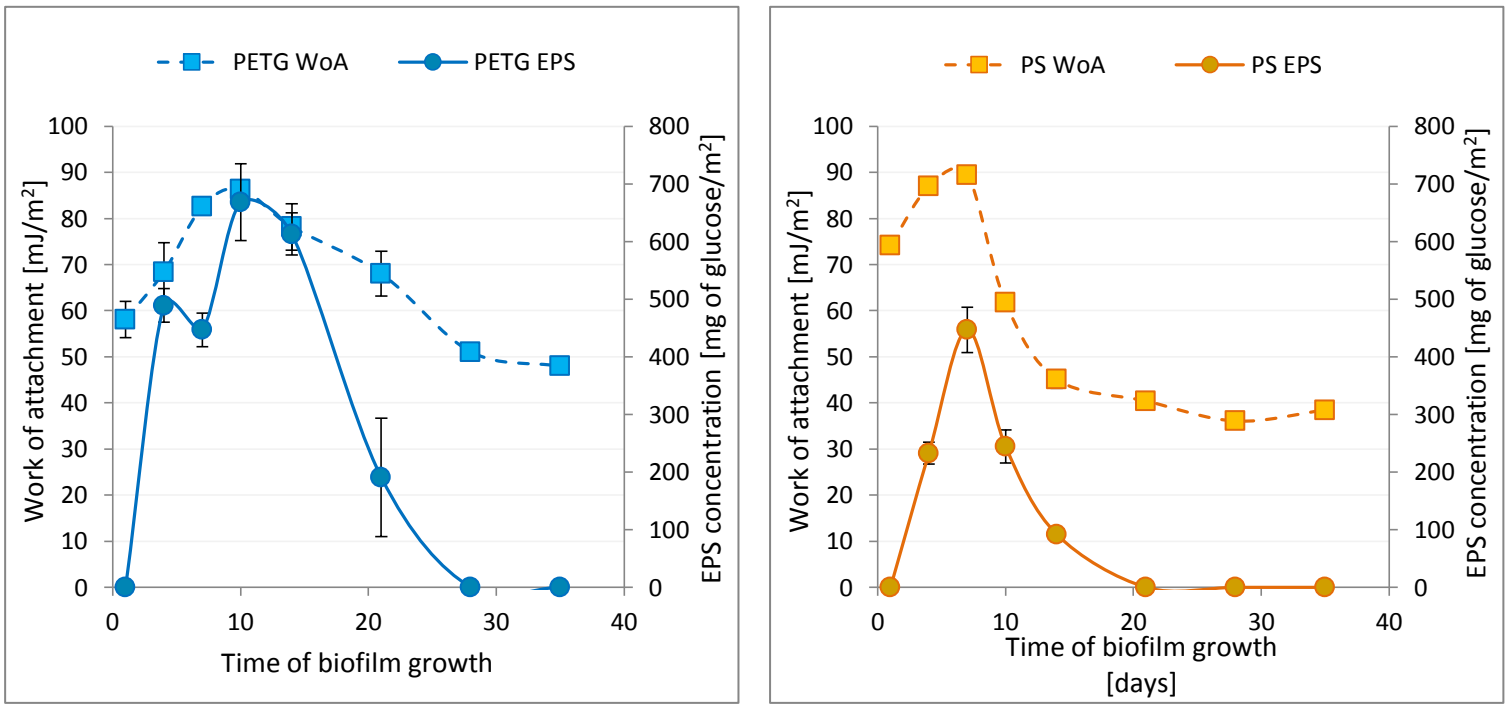
The work required to remove loose cell was much lower than the work to remove EPS-embedded cell. According to the thermodynamic theory, it was much easier to detach cell which did not secrete polymeric substances: 17.56±1.26 (PETG) and 18.47±1.40 (PS) mJ/m^2 compared to 58.07±3.96 (PETG) and 74.16±0.80 (PS) mJ/m^2. This relationship was observed for both PETG and PS materials. The work of attachment gradually increased up to day 10 and 7 for *Stichococcus* sp. biofilm development on PETG and PS, respectively. The maximum values were 86.35±0.23 for PETG and 89.43±0.26 mJ/m^2 for PS. After one week of biofilm growth, the work of attachment started to drop. The thermodynamic model suggests that it is easier to remove the cells when the biofilm reaches exponential and stationary growth phase, which was also indicated by shorter experimental removal energy. Cells from mature

biofilms formed on both PS and PETG was easier to detach after five weeks of growth as work of detachment decreased to 38.45±0.66 and 47.97±1.16 mJ/m^2, respectively.

The thermodynamic equation also suggests that the strength of adhesion on both substrates was very similar throughout biofilm development. The maximum work of detachment on both plastic materials was almost identical; however it increased and decreased more rapidly on PS than on PETG.

Table 13 Work of attachment of *Stichococcus* sp. growing biofilm

Time [days]	*Stichococcus* sp. on PETG				*Stichococcus* sp. on PS			
	γ^{total} [mJ/m^2]	$\gamma^{dispersive}$ [mJ/m^2]	γ^{polar} [mJ/m^2]	WoA, ΔG_{adh} [mJ/m^2]	γ^{total} [mJ/m^2]	$\gamma^{dispersive}$ [mJ/m^2]	γ^{polar} [mJ/m^2]	WoA, ΔG_{adh} [mJ/m^2]
0	48.69±3.99	18.67±2.07	30.02±1.93	17.56±1.26	48.69±3.99	18.67±2.07	30.02±1.93	18.47±1.40
1	40.39±1.20	34.15±0.41	6.23±1.61	58.07±3.96	39.52±0.43	37.51±0.23	2.01±0.20	74.16±0.80
4	37.53±0.30	34.70±1.89	2.83±1.58	68.43±6.33	40.26±0.59	40.08±0.58	0.18±0.01	86.99±0.00
7	38.83±1.01	37.58±1.00	0.25±0.01	82.56±0.1	37.48±0.03	37.45±0.04	0.03±0.01	89.43±0.26
10	36.82±1.44	36.81±1.44	0.01±0.00	86.35±0.23	43.37±1.24	37.49±1.20	5.88±0.04	61.74±0.22
14	36.23±0.77	35.41±0.09	0.82±0.68	78.19±5.02	46.16±0.08	32.67±0.95	13.49±1.03	45.13±1.99
21	42.05±0.63	38.86±0.74	1.82±0.91	68.03±4.82	49.07±0.15	32.63±0.48	16.44±0.63	40.40±1.09
28	40.87±1.40	31.81±1.33	9.06±0.07	51.08±0.27	50.91±1.06	31.69±0.56	19.22±0.50	36.07±0.54
35	47.17±1.23	35.78±0.49	11.39±0.75	47.97±1.16	49.738±0.73	32.10±0.99	17.64±0.26	38.45±0.66



A **B**

Figure 41 WoA in relation to EPS excretion by *Stichococcus* sp. on PETG (A) and PS (B)

As showed in Figure 41, the work of attachment increased with increasing amount of EPS on both PETG and PS. It is clearly visible, that presence of EPS significantly increases the work required to separate cell from substratum. It underlines the importance of EPS, which have been proven to play an essential role in cells attachment.

Park *et al.* [286] experimentally investigated the influence of EPS presence on biofilm formation in their work on activated sludge. The number of attached microorganisms was two times lower without EPS. Only $1.8 \cdot 10^8$ treated cells/cm^2 adhered in comparison to $3.3 \cdot 10^8$ untreated cells/cm^2 which attachment was supported by EPS. Similarly, in this study, the TD model suggested that it was much easier to separate cell from the surface when no biofilm was formed, as only 17.56±1.26 and 18.47±1.40 mJ/m^2 WoA was required instead of 58.07±3.96 and 74.16±0.80 mJ/m^2, respectively. EPS secretion was especially important in the case of PETG, as this material lacked

of any textural features. The gelling properties of EPS helped *Stichococcus* sp. cells during biofilm formation. Initial amounts of EPS and SMP on PETG were higher than on PS material, presumably due to the fact that the culture needed to secrete more EPS to help its attachment on smoother PETG. SEM images of *Stichococcus* sp. biofilm on PETG showed that the matrix was not homogeneous and cells were separated in microbial aggregates (Figure 28 B, Chapter 5.4.1). This is also echoed by the number of cells attached on PETG after the first four days of biofilm cultivation, lower than the number of cells attached on rougher PS. When the culture started producing EPS (4th day of cultivation), the improved nutrients transportation [384] allowed a better growth and higher number of cells.

6.4.2.2 WoA in relation to experimental detachment

Experimental detachment was not correlated to the theoretical work evaluated using the thermodynamic equation. However, the work of attachment is the work required to separate single cells from the substrate, not to remove the whole biofilm. The experimental energy of detachment is related to the growth phase of the whole biofilm, while theoretical attachment is dependent upon the level of cells aggregation.

Work of attachment decreased with increased biofilm polarity. Polarity of strains have been reported as an important parameter to be considered in primary attachment of cells, but so far no such relation was showed for growing biofilm. Both WoA and polarity did not match the experimental attachment data. This is mainly due to the fact that the thermodynamic model focuses on physico-chemical properties of the system and no biological interactions are taken into account.

6.4.2.3 Change in biofilm properties

In 2013 Janjaroen *et al.* [385] investigated the attachment mechanism of *E. coli* on polyvinyl chloride. Diiodomethane contact angle was measured during biofilm growth for 27 weeks. Their results indicated that the diiodomethane contact angle decreases with time, and the dispersive component of surface free energy, γ^{LW} increases with time [385].

Table 14 Water, diiodomethane and ethylene glycol contact angles of biofilm during 35 days cultivation period

Day	Water contact angle, θ_W [°]	Diiodomethane contact angle, θ_D [°]	Ethylene glycol contact angle, θ_E [°]
1	73.37±4.96	47.03±1.70	50.23±3.67
4	85.50±6.08	48.69±3.51	53.24±3.25
7	111.72±1.01	46.62±2.41	61.79±2.94
10	102.80±4.79	45.04±3.34	69.55±2.48
14	96.72±8.19	52.01±7.38	52.72±8.57
21	84.70±1.78	38.25±0.56	52.34±5.88
28	63.75±1.51	46.35±2.46	60.16±6.81
35	54.02±3.20	35.80±2.70	55.47±4.34

As shown in Table 14, diiodomethane contact angle was constant through cultivation period (θ_D=44.97±3.97 for PETG and θ_D=41.07±1.99 for PS). Moreover, SFE dispersive component of biofilm did not change during microbial growth (γ^d=35.76±2.33 for PETG and γ^d=35.20±3.26 for PS), as it can be seen in Table 13. Assuming γ^d is equivalent to γ^{LW} [355], there was no correlation between γ^d measured in this study and γ^{LW} measured by Janjaroen *et al.* [385]. Discrepancy between Janjaroen *et al.* results and data showed in this chapter may be caused by the fact that in this study, biofilm growth was monitored over shorter period of time (5 weeks) in comparison to Janjaroen *et al.* work (27 weeks). Janjaroen *et al.* were performing measurements every 2 weeks that is why eventual oscillations in diiodomethane contact angle value could be missed. In addition, γ^{LW} was calculated based only on one reference liquid, which is not enough to obtain accurate results. Three-liquid method or at least two-liquid method, where one polar and one non-polar liquids are used, is recommended to obtain reliable results [386].

The change in physical properties of the biofilm was observed mainly as an initial increase in water contact angle followed by a decrease around 7th day of cultivation and initial decrease in biofilm polarity followed by increase starting at 7th (PS) and 10th (PETG) day of cultivation. Change in polarity and water contact angle is presented in Figure 42.

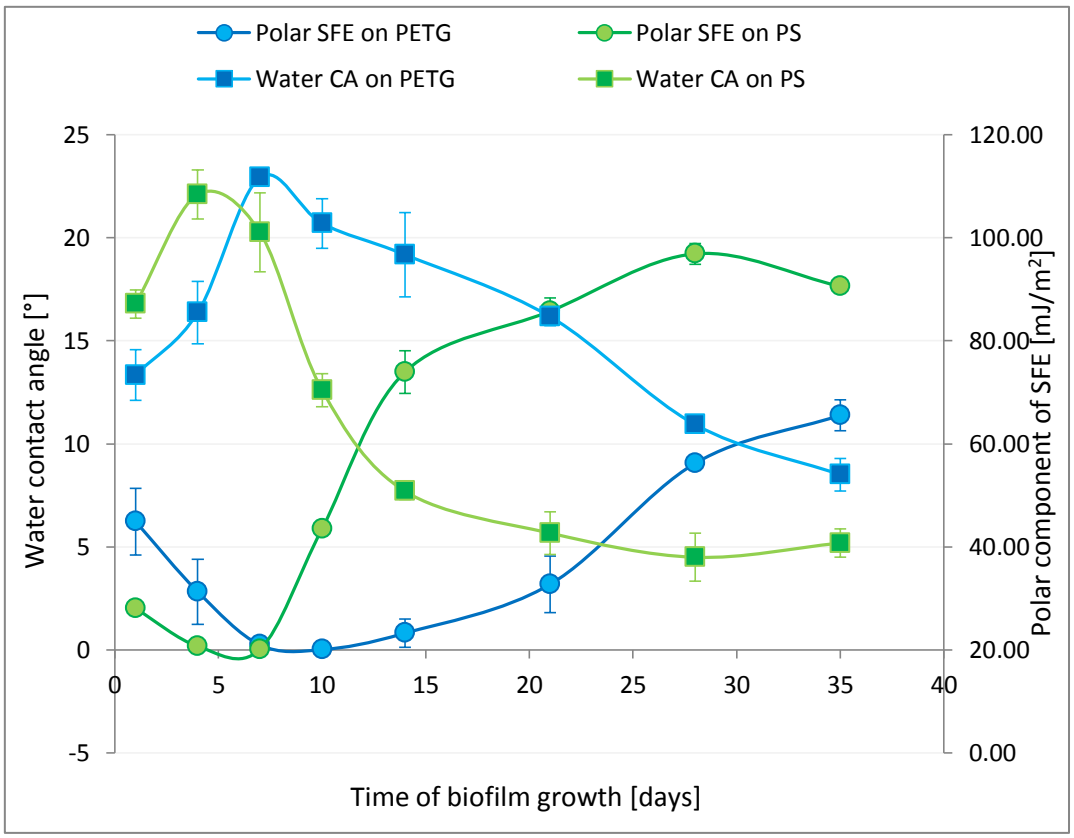


Figure 42 Physical properties of biofilm over time

Change in physical properties of biofilm differs from the study conducted by Janjaroen *et al.* [385] because their measurements were based on limited number of reference liquids. Therefore, the study of Janjaroen *et al.* was not able to fully reflect the behaviour of biofilm during growth. To the best of author's knowledge, there are no other studies on changing physical properties of developing biofilm.

The physical properties of the biofilm on both surfaces were connected to its growth phases: lag, exponential and stationary. The biofilm polarity decreased during the lag phase (1st week of cultivation), from 6.2±1.6 to 0.031±0.007

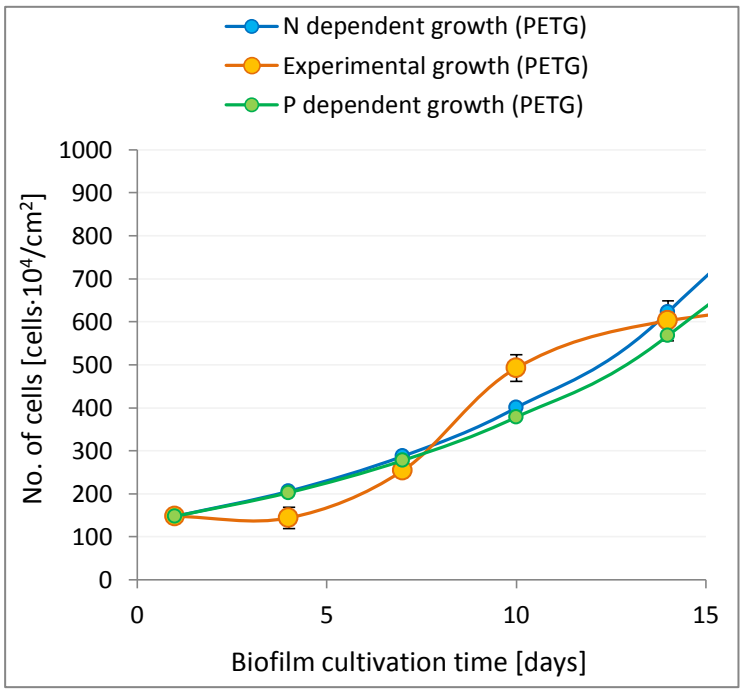
mJ/m^2 on PETG and from 2.0±0.2 to 0.019±0.004 mJ/m^2 on PS. At the same time, the water contact angle increased and biofilm became more hydrophobic. When exponential growth started, the polarity increased, while the water CA started to drop. This trend was observable during the remaining course of the biofilm growth. Mature biofilm became more hydrophilic, especially on PS, where the water CA dropped to 40°. *Stichococcus* sp. was able to switch between hydrophobic and hydrophilic phenotype, which was also observed by Borecka-Melkusova and Bujdakova [150] on their work on *Candida albicans* and *Candida dubliniensis*, and by Bujdakova *et al.* [223] on *Candida albicans*. The hydrophilic surface of mature biofilm inhibited the co-aggregation of more species from the liquid medium. In addition, the change in hydrophilicity of biofilm had a supplementary impact on its sloughing. At the end of the growth cycle, the biofilm started to release special enzymes able to degrade EPS [332]. Assuming that the amount of secreted EPS was reversibly proportional to the biofilm polarity, the EPS degradation would cause an increase in the polar SFE of the biofilm. The change in biofilm hydrophobicity could be explained by self-induced detachment, observed for mature biofilm.

6.4.3 Monod model in relation to biofilm growth

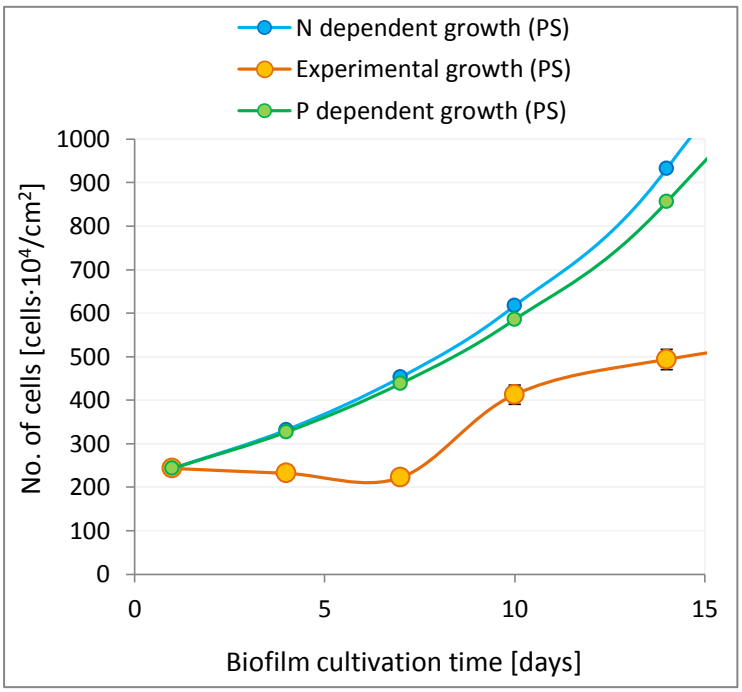
The results of *Stichococcus* sp. and *C. saccharophila* growth predicted by Monod kinetics are presented in Table 29 and Table 30, respectively (Appendix D.7). Monod model has wide applications in description of microbial suspended growth. The model originally was not designed for biofilm growth predictions, although it was tested to evaluate its applicability in sessile state.

Both TN and TP were important components for microbial growth. Cells did not reach decreasing rate of growth predicted by Monod for system that is nutrient-dependent. The shape of growing community resembled constant exponential growth for the whole 35 days of cultivation, therefore system was not nutrient-restricted. Nevertheless, the specific structure of biofilm may cause local nutrient-deficiency and lead to decrease in biofilm growth rates.

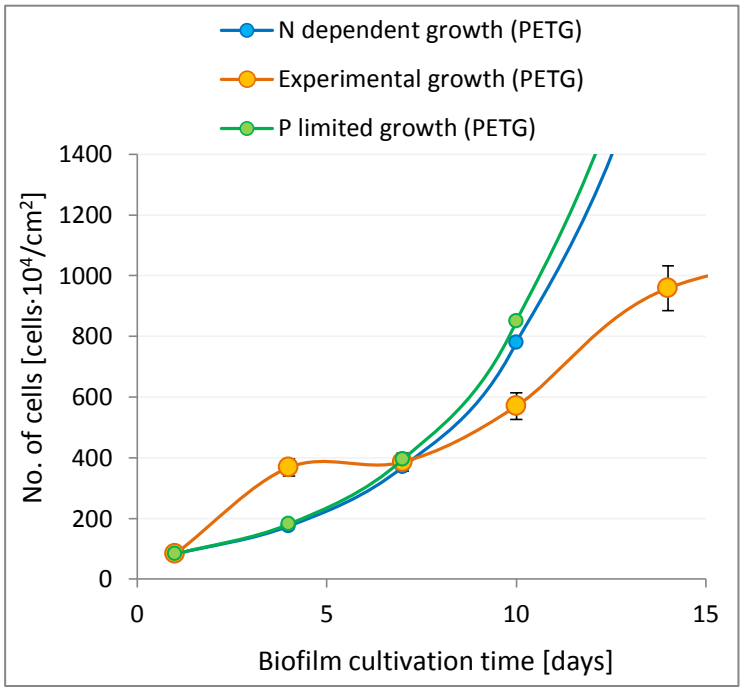
Relation between experimental data and Monod-predicted growth for initial biofilm development is presented in Figure 43.



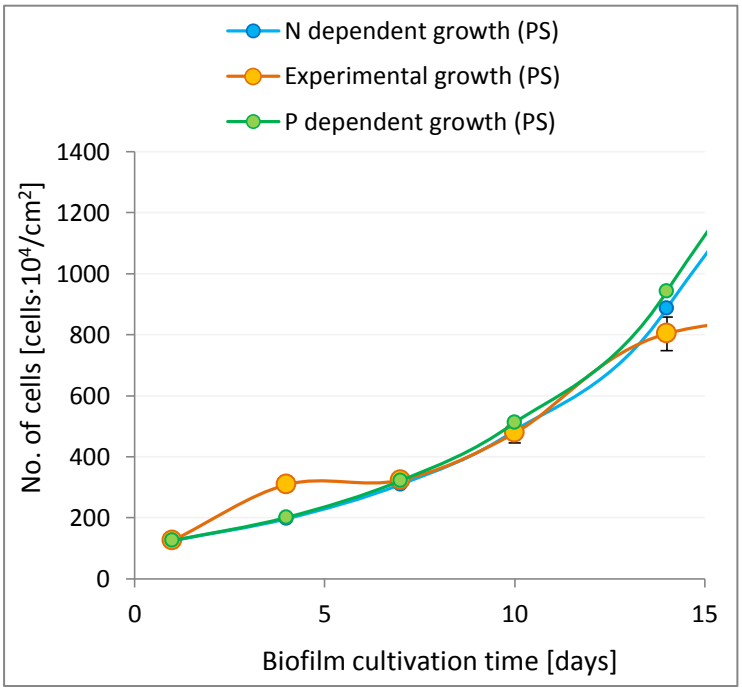
A *Stichococcus* sp. (PETG)



B *Stichococcus* sp. (PS)



C *C. saccharophila* (PETG)



D *C. saccharophila* (PS)

Figure 43 Experimental growth of microalgal biofilm compared to N- and P-dependent growth calculated by Monod equation

A characteristic fluctuation in number of cells was observed in all experimental data (Figure 43). Fluctuation in empirical biofilm density was caused by many

factors that are not taken into account in simple Monod model: influence of shear forces on detachment rate, balance between growth and death rates and availability of nutrients in boundary layer. Nevertheless, those factors did not significantly impact initial biofilm growth. The Monod kinetics was able to predict initial biofilm development with similarity from 60 to 86%.

At stationary growth phase and death phase, a significant difference between experimental and theoretical data was observed (Appendix D.7). According to the Monod kinetics, in planktonic state cells are able to multiply up to $80.2 \cdot 10^6$ cells/cm^2 on polystyrene after 35 days of cultivation. However, number of cells grown in sessile state was equal to only $2.86 \pm 13 \cdot 10^6$ cells/cm^2. Monod model did not take into account specific mechanisms governing nutrient absorption in biofilms, in particular the nutrient starvation that takes place due to decreased access to nitrogen and phosphorous by cells encapsulated in deeper parts of biofilm matrix. Phenomenon of self- detachment induced by cells as a defence against nutrient starvation was also not taken into account. Furthermore, no shear forces of moving liquid were included into the model; medium movements take place even in standing cultures.

Nevertheless, Monod model is a useful tool for predicting initial sessile growth, when biofilm thickness is too small to significantly impact nutrient absorption and availability. Small data input is a very big advantage, particularly in design of biofilm reactors [368]. However, Monod model is not applicable for mature biofilms. Monod limitations regarding biofilm maturation mostly comprise nutrient availability profile, the steady-state growth phase and self-induced sloughing.

6.5 Conclusions

Thermodynamic model is not a good tool for predicting quantity of initial attachment. However, TD model is able to give an overall insight into propensity of cells to attach, such as which strain would attach better or the worst material for attachment. Those insights can significantly help in material and strain selection in biofilm bioreactors.

During cultivation, the surface properties changed and physical and chemical interactions were no longer the primary processes governing biofilm development. Biological interactions dominated during biofilm development, and those were not taken into account by the models considered in this study.

Work of adhesion did not match energy required for experimental detachment. To obtain more appropriate comparison between theoretical and empirical detachment, another method allowing single cell removal should be introduced. The alternative is to evaluate work required to detach whole biofilm. However, such model would involve more complex approach than TD model.

The work of attachment increased with the amount of EPS excreted, indicating that more energy will be required to remove a single cell from EPS-embedded biofilm. The release of EPS influenced the physical properties of the biofilm and surface hydrophilicity increased with EPS degradation. Increase in biofilm hydrophilicity at the end of growth cycle inhibited further co-aggregation of microbial species to nutrient-deficient environment.

Monod kinetics designed to describe growth at planktonic state was a good prediction tool for initial biofilm growth, giving helpful indications for cyclic biofilm-based systems. However, it failed to describe the sloughing phenomenon of mature biofilm. For mature biofilms, more advanced mathematical model would need to be applied for estimations on biofilm growth, which would possibly incorporate nutrient availability within biofilm with EPS production and breakage.

7 Non-suspended aeroterrestrial cultivation of *Chlorella saccharophila*: Humid Biofilm-Bioreactor (HBBR)

7.1 Abstract

To address some of the sustainability issues associated with microalgae production, a Humidity Biofilm Bioreactor (HBBR) was designed. HBBR performance was compared with a commercially available suspended photobioreactor (SPBR) and submerged biofilm bioreactor (SBBR). *Chlorella saccharophila* microalgal strain was cultivated on polystyrene (PS) carrier substrate in HBBR and all the reference reactors.

The maximum growth rate inside the HBBR was 0.14 day^{-1} for *C. saccharophila*, two times higher than in conventional SBBR and commercially available SPBR. HBBR reached the maximum biofilm density in a shorter time period. Furthermore, the novel cultivation system had additional benefits. Light distribution within the reactor was not disrupted by free-floating cells. Therefore culture did not suffer from self-shading, as the optimum biofilm thickness was small. The water consumption was significantly lower in comparison to reference reactors and HBBR did not suffer from lag phase. Finally, the biomass concentration of the end product was very high (345.3±68.6 g of biomass/L of water) and besides scraping, it did not require additional post-processing.

7.2 Introduction

7.2.1 Research needs

The great potential of microalgae has been recognised in many industrial sectors, from removal and remediation to production of biofuel, feed and added value compounds. Microalgae can be cultivated in order to obtain various compounds, which can be used in pharmaceutical [387], nutraceutical [3], petrochemical [388], aquaculture [389], aerospace [390], agriculture [391] or food [4] industries. Microalgae do not compete with the food chain, are relatively easy to grow, as they do not require fertile soil [392] [393]. Current cultivation systems are mainly based on microalgal cultures suspended in water, in either

open ponds or closed bioreactors. Production of microalgae for industrial applications is still relatively problematic as there are many factors that need to be controlled in order to obtain stable and satisfactory growth. Bioreactor cultivation often requires axenic conditions which can be quite challenging and expensive at pilot-scale or industrial-scale [394].

Other potential issues associated with suspended growth are low cell density and water consumption. The volume of water used during suspended cultivation processes is quite high. It has been estimated that around 4000L of water are required to produce 1kg of algae-derived biodiesel [62]. Expensive post-growth steps, such as harvesting, are often required to isolate the final product [5]. Harvesting for example can contribute up to 1/3 of the total production costs [39]. One potential answer to the problem would be to cultivate cells at higher concentrations but higher concentrations of cells can have an impact on light distribution inside the reactor, decreasing algae growth. Both open and closed systems suffer from self-shading leading to decrease in biomass yields [395]. Light penetration in the systems is limited and intensive mixing needs to be applied in order to evenly distribute the light through the culture, which result in additional costs.

7.2.2 Non-suspended systems for microalgal cultivation

Non-suspended cultivation can help to address some of these issues. Biofilm-based growth is a well-established cultivation method in which microalgae are grown attached onto solid substrates in a liquid environment [65]. The main advantage of these systems is the reduction in time and energy needed for the dewatering step or the complete elimination of this step [50]. During non-suspended cultivation microalgae can be either encapsulated into a matrix (enclosure methods) or grown concentrated onto a specific surface area (non-enclosure methods) [8]. Enclosure methods are currently tested for tertiary treatment of wastewater at pilot scale [396] but they are too expensive to be implemented for high volume/low cost growth due to the high cost and complexity of retrieving microalgae from the encapsulation matrix [50].

Many authors have described algae growth in biofilm-based systems in relation to the medium used (synthetic [21] or wastewater [18]) or to the different materials for attachment (polycarbonate, polyethylene, stainless steel mesh [18], acrylic, glass, polystyrene, cotton [21], polypropylene [33], jute [32]). Reported biomass productivity achieved in attached growth systems varies from 0.45 g [180] up to 40.8 g [22] of biomass/m^2/day. Only a few biofilm-systems have been proven at larger scale [18] [32].

Other unconventional biofilm-based growth methods in liquid media have been reported in patents. Picard G. (2014) [397] suggested a cultivation method on a fabric mat in which the liquid medium/nutrients was delivered by capillary action and the cell were harvested by pressing out the biomass. Gross and Wen (2014) [398] designed a Rotating algal biofilm (RAB) reactor using cotton belt. In the reactor the belt, used for cell attachment and only partially dipped into the medium, was moved by rollers to ensure a constant contact between the microalgae and the medium. Finally, Zimmermann U. (2000) [399] proposed to grow microalgae on an anchoring device continuously wetted by a thin film of liquid medium in a high humidity environment.

In our study, algae biofilms were grown under aeroterrestrial conditions. Once established, the biofilm was maintained in a humid atmosphere (relative humidity range 75-95%) and their growth monitored over time. The advantages compared to a liquid system include: (i) lower water consumption; (ii) lower lighting requirements; (iii) lower energy consumption. In addition, the biofilm surface could be set at an angle to allow self-harvesting.

Chlorella saccharophila was grown in humid-aeroterrestrial conditions on polystyrene (PS) for 42 days, without re-growth cycles. Growth in the humidity bioreactor (HBR) was compared with two reference reactors, using the same strain and material: submerged biofilm bioreactor (SBBR) and suspended photobioreactor (SPBR).

7.3 Materials and methods

7.3.1 Strains and conditions prior to growth

Species selection was made based on the results on primary attachment of four microalgal species and nine materials (Chapter 3) and short growth trials described in Chapter 4. Preliminary tests inside the reactor allowed the identification of the best strain-substrate couple. Based on selection criteria given in Appendix B, *Chlorella saccharophila* CCAP 211/57 was chosen for the investigation. The freshwater microalgae *Chlorella saccharophila* CCAP 211/57 was acquired from the Culture Collection of Algae and Protozoa (CCAP), Aberdeen - UK. Prior the experiment, the strain was cultured in liquid Bold Basal media using an Innova®44 incubator shaker at 22°C, 180 RPM, 3110 lux (42 µmol/m^2/s) light intensity. The algae were grown in the different reactors (HBBR, SBBR and SPBR) for a total period of 6 weeks.

7.3.2 Substrate material

Material chosen for the novel cultivation system and non-suspended reference reactors was polystyrene (PS) plastic. Material selection was made based on the experiments described in Chapter 3 and 4. The detailed description of selection process is given in Appendix A. PS was obtained from FisherBrand, UK. The size of each material sample was 8cmx 3cm. Prior to inoculation, PS substrates were cleaned with propanol.

7.3.3 Humid Biofilm Bioreactor

The system was designed (Figure 44) to maintain steady humidity inside the reactor and to deliver an appropriate amount of Bold Basal medium, via nebulisation, to meet the microalgal nutrients requirements [134]. Growth tests were performed inside a 100 L capacity glass tank. Substrates with microalgae biofilm were accommodated on aluminium grates suspended on hooks inside the reactor. Two fluorescent lamps positioned at the top of the reactor were used as a light source. Microalgae biofilm was grown under 12:12 light

illumination (fluorescent lights) at 3110 lux (42 µmol/m^2/s). The temperature inside the reactor was maintained at 22°C.

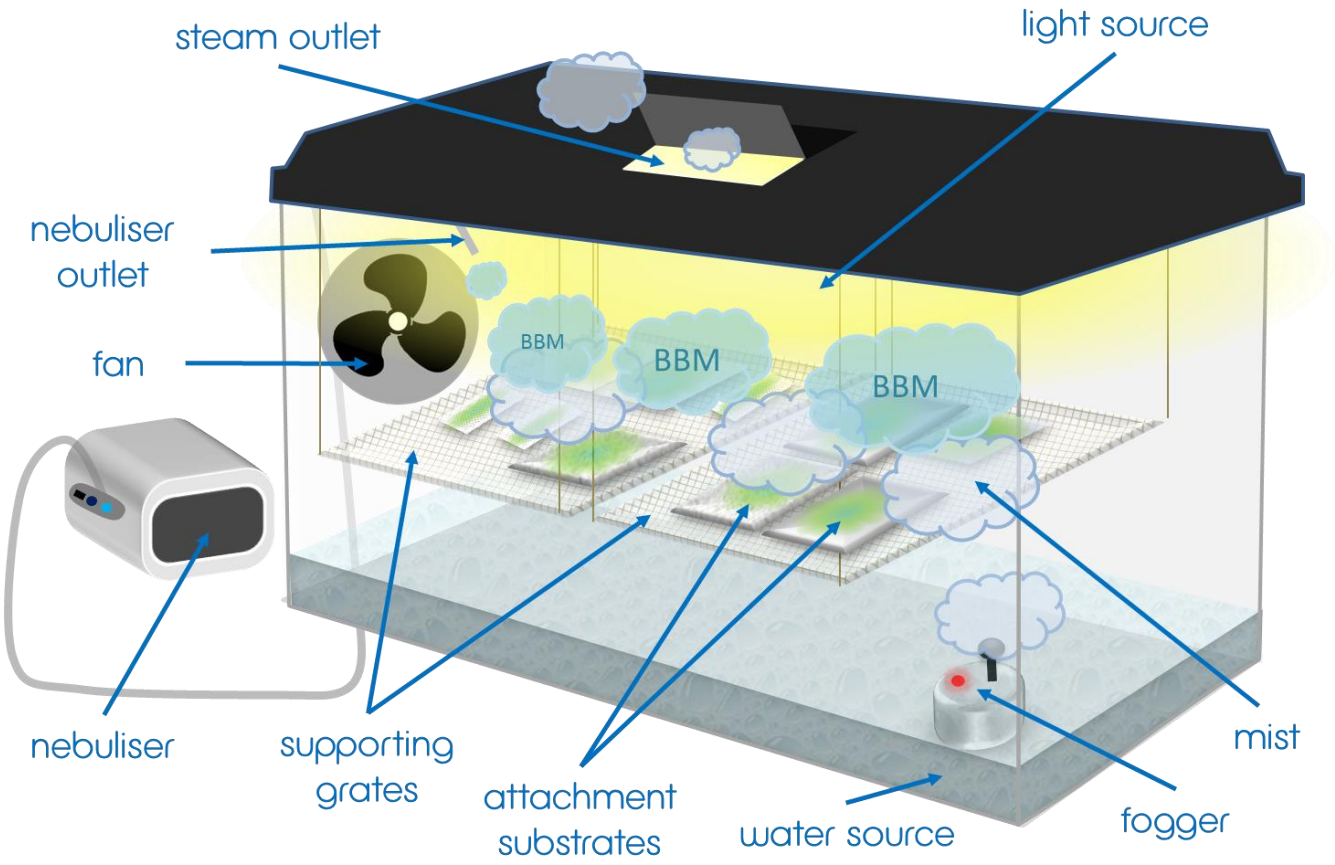


Figure 44 Schematic of the HBBR

The humidity inside the HBBR was maintained at 75-95% relative humidity (RH). The relative humidity level was controlled by a fogger located at the bottom of the reactor (Maplin, UK). BBM was delivered by a nebuliser (Omron NE-C28P, Omron Healthcare, UK) at a total rate of 0.25 ml/min. Even distribution of humidity within the reactor was controlled by a fan placed on the reactor wall. The steam outlet, minimised the problem of oversaturation.

7.3.4 Reference Photobioreactors

Two laboratory scale photobioreactors were used as reference to assess and compare microalgae growth under different cultivation conditions (non-suspended and suspended):

- Submerged biofilm non-suspended bioreactor (SBBR). Microalgae were cultivated in batch mode following the method reported in Schnurr *et al*. [19]. The reactor consisted of a 10L glass tank, containing Bold Basal media and maintained at 22°C, with no flow control or recirculation. The inoculated samples were arranged horizontally inside the polypropylene container and covered with liquid medium.

- Suspended photobioreactor (SPBR). Commercially available Innova®44 incubator shaker was used as a suspended third reference photobioreactor. 100ml of sterile Bold Basal media in 250ml Erlenmeyer flasks, was inoculated with C*. saccharophila* and placed in the incubator at 22°C, 180 RPM, 3110 lux (42 µmol/m^2/s) light intensity.

7.3.5 Growth conditions

BBM was used as nutrient source in HBBR, SBBR and SPBR. BBM was prepared according to CCAP media receipt [206]. 100 ml of standard BBM (41.17 mg/L of nitrogen and 50 mg/L of phosphorous) was supplied to SBBR weekly, to support microalgal growth. In SPBR, 5 ml of concentrated BBM (824 mg/L of nitrogen and 1000 mg/L of phosphorous) was supplied weekly to the culture, so that the amount of nutrients added to every reactor was the same.

Microalgae were cultivated on substrates under 12:12 LED light illumination by fluorescent lamps at 42 µmol/m^2/s light intensity. Temperature was maintained at 22°C inside all reactors.

7.3.6 Inoculum preparation

Prior to experiments, *C. saccharophila* was grown in Innova®44 photobioreactor (BBM, 12:12 illumination regime, 42 µmol/m^2/s light intensity, 22°C, 180RPM). Starting cultures of *C. saccharophila* grown in liquid were used as an inoculum for HBBR and SBBR systems and for growth in SPBR. All cultures aimed to have a similar initial cell concentration (1.6±0.2 cells·10^6/ml). PS substrates were arranged inside a polypropylene container containing the pre-grown *C. saccharophila*. The container was closed and maintained in the dark to promote cell deposition. After overnight inoculation, samples were removed from the

container and transferred to the non-suspended reactors. The amount of cells on those samples and the number of cells inside SPBR were counted and used as time zero.

7.3.7 Growth measurement

Three samples were collected every 7 days from each photobioreactor (HBBR, SBBR and SPBR) for a total period of 42 days. In the case of HBBR, additional measurements were taken at day 2 and 3 of cultivation to better illustrate the cell behaviour inside the novel bioreactor.

To evaluate biofilm growth, each substrate was placed inside a buffer solution (10ml, pH=7.4) and sonicated for 5-30 minutes until the biofilm was removed. Absorbance of sonicated samples was recorded at 540 nm wavelength by Jenway 6800 Double Beam UV/ViS Spectrophotometer. The value of absorbance was recalculated into number of cells with the use of standard curve, constructed prior commencing the experiment.

In order to determine the moisture content in the biomass, weight measurements were performed according to laboratory analytical procedure introduced by Wychen and Laurens [400]. Substrates were weighted before inoculation (no biomass), after removal from reactor (wet biomass weight) and after drying (dry biomass weight). To determine dry biomass weight, all samples were dried inside the oven at 60°C temperature for 18 hours.

7.4 Results and discussion

7.4.1 Growth in HBBR and reference reactors

Growth of *Chlorella saccharophila* inside HBBR and SBBR is presented in Figure 45. Growth in SPBR expressed in cells·10^4/ml was shown for comparative purposes. All samples in non-suspended reactors had the same inoculum density equal to 1.6±0.2 cells·10^6/cm^2. The number of cells on the 1st day of cultivation corresponded to the number of cells in every sample after inoculum and was the same for all non-suspended reactors.

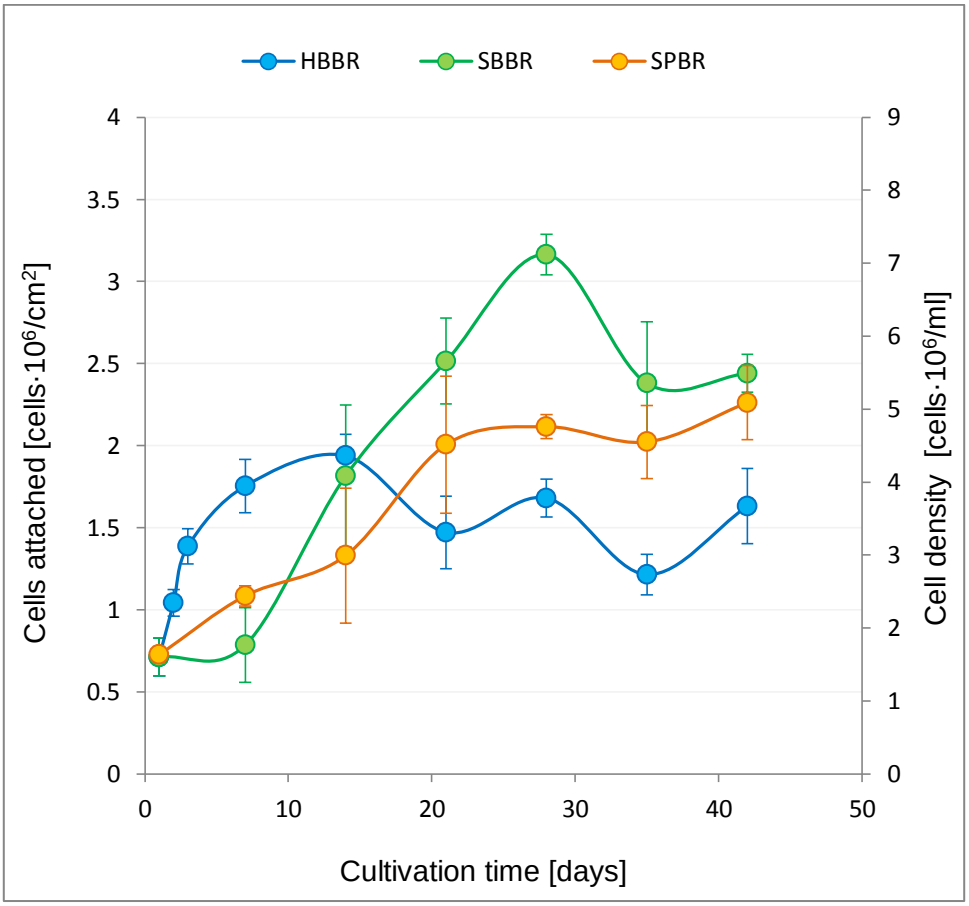


Figure 45 Growth of *C. saccharophila* inside HBBR, SBBR and SPBR

Biomass density in HBBR increased exponentially until 2nd week, followed by sloughing. Sloughing is a rapid, massive removal of microalgal cells [23]. Lack of lag phase was also observed in SPBR. In SBBR, lag period lasted for 7 days, after that time microalgae slowly started to grow. In lag phase cells are metabolically active, however, they are not able to divide yet [401]. According to Rolfe *et al.* [402], lag growth phase is poorly understood. Regulatory mechanism governing the equilibration process, which was described by Monod [58], is still unidentified. Extensive studies on lag and exponential growth phases showed that it is possible to predict the duration of lag phase for certain bacterial species, however for the same environmental conditions only [403] [404]. As each reactor in this study was characterised by different properties such as medium movements (SPBR) or physical state of nutrients (HBBR), it was not possible to compare length of lag phases between reactors. Therefore,

it was expected that the duration of lag phases of *C. saccharophila* varied between reactors.

Exponential growth phase lasted till 14th day in HBBR, 21st day in SPBR and 28th day in SBBR. The maximum number of cells in HBBR was reached at 14th day of cultivation and was equal to 1.9±0.1 cells·10^{6}/cm^{2}. Highest cell density was obtained in SBBR (3.2±0.1 cells·10^{6}/cm^{2}), however it took almost 28 days for the culture to achieve it. Lag phase in SBBR system increased the total time required to achieve the maximum biofilm density. Therefore, it is possible that the re-growth cycle in SBBR would be more effective than the re-growth cycle in HBBR. Re-growth cycles are usually characterised by shorter lag phase due to the same environmental conditions. Moreover, they have higher growth rates in comparison to start-up cycle, as cells are harvested at exponential growth [405].

All biofilm-based systems were characterised by a high peak in growth density followed by a spontaneous decline in growth. Meanwhile, *C. saccharophila* in SPBR was able to maintain the balance between growth and detachment of cells for a longer period of time. However, culture growing in liquid medium does not undergone sloughing or abrasion. In the case of nutrient depletion, cells are free to search for a new environment rich in nutrients. Even immotile cells can move thanks to the Brownian motion. In contrast, cells growing in biofilm are embedded within its matrix. Biofilm community usually trigger auto-detachment after some period of growth [332]. That is why, at some point in the cultivation, a rapid decrease in number of cells was observed in all non-suspended reactors. Large biofilm aggregates were detached from biofilm. This phenomenon is illustrated in Figure 46.

A B C

Figure 46 Development of *C. saccharophila* biofilm. From left: after inoculation (A), at exponential growth (B), entering sloughing phase (C).

Further investigations on re-growth cycle in HBBR could show the full potential of the system, especially since the start-up phase of biofilm growth was proven to have lower productivity than subsequent growth-harvest cycles [7].

7.4.2 Growth rates comparison

Growth rate is an intensive property, which does not depend upon certain number of microalgal cells, but describes cell division. Therefore, growth rate is perfect for comparison between reactors that have different unit of cellular density. In non-suspended reactors, biofilm density is expressed in cells per cm^2 of the material. In suspended systems, the number of cells in the liquid culture is usually expressed per ml of the culture. It is not possible to compare those two numbers together. It would be possible only when packing density of non-suspended systems would be known. Packing density describes compactness of substrates in system and gives the maximum number of substrates per volume unit. However, in this study it was not possible to evaluate packing density, as more extensive experiments would need to be performed. Thus, growth rates in HBBR, SBBR and SPBR were compared and showed in Figure 47.

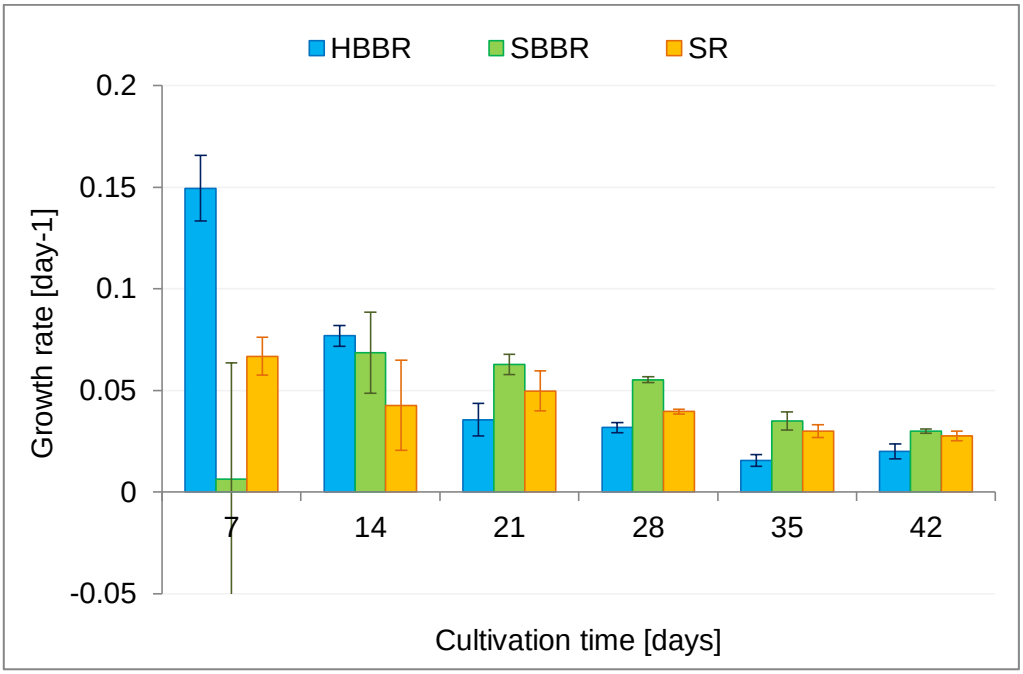


Figure 47 Growth rates of *C. saccharophila* in HBBR, SBBR and SPBR

At 7th day of cultivation, cells multiplication in HBBR was the most intensive among all tested reactors. It was two times higher than in SPBR: 0.15±0.02 compared to 0.07±0.01 day^{-1}, respectively. However, after a few days the growth rates inside the HBBR system slowly started to decrease. The gradual drop in growth rates was observed through the whole cultivation period. At the same time, *C. saccharophila* cells in SPBR maintained relatively steady growth rates at 14th, 21st and 28th week of cultivation: 0.07±0.01, 0.04±0.02 and 0.05±0.01 day^{-1}, respectively. Starting from 21st day of cultivation, growth rates in SBBR were the highest in comparison to SPBR and HBBR. In general, the growth of *C. saccharophila* cells varied between reactors during the lag and initial exponential growth rate. Nevertheless, starting from the 3rd week of cultivation, division of cells was comparable for tested reactors. From above comparison it was clear that cells in HBBR intensively multiplied in the first two weeks of cultivation. The growth in remaining reference reactors was more uniform throughout the whole cultivation period.

7.4.3 Biomass concentration

High moisture level is a common problem associated with microalgal cultivation methods [406]. Large amounts of water are used particularly in suspended systems and are associated with low dry biomass content. The product with the highest mass fraction of biomass to water is desirable. Cao *et al.* [407] showed that it is possible to produce biodiesel from *Chlorella pyrenoidosa* biomass of high water content. However, the production yields were much lower than for pure biomass. Biomass yields decreased from 91.4% to 10.3% for water content from 0 to 90% at 90°C. The biomass content in SBBR and SPBR was much lower than 10%. Non-suspended reference reactor (SBBR) was able to achieve much higher biomass concentrations in comparison to SPBR: 97.4±32.4 g of biomass per L of water compared to 2.9±0.6 g/L, respectively. Similar relation between non-suspended and suspended reactors was observed in other studies. *Nannochloropsis* sp. cultivated in suspended glass photobioreactor reached biomass concentration up to 2.23 g/L [408], very similar to this study. Lal and Das [409] cultivated *Chlorella* sp. in airlift photobioreactor, achieving maximum biomass concentration equal to 1.25 g/L after 9 days of cultivation. *Scenedesmus* sp. grown by Hakalin *et al.* [410] in Erlenmeyer flasks reached 1.3 g/L. In general, microalgae grown in open systems achieve very low biomass concentration, around 0.5 g/L [40], while in enclosed system it is possible to achieve slightly higher biomass concentration, up to 10 g/L [41]. Biofilm bioreactors are able to achieve significantly higher biomass concentrations, as cells are grown concentrated in certain areas. Blanken *et al.* [7] grew *Chlorella sorokiniana* on rotating disks, reaching between 143 and 192 g/L biomass concentration. Another biofilm-based system introduced by Christenson and Sims [32] was also able to produce biomass of high concentration. Mass fraction of mixed culture biomass to water was equal from 120 to 160 g/L [32]. Biomass concentrations in other biofilm-based systems were also very high and could be compared with the results obtained in this chapter. Biomass yields obtained by Gross *et al.* [21] in their rotating algal biofilm reactor were comparable to the results from SBBR system: 97 g/L and

97.4±32.4 g/L, respectively. Specie grown in Gross *et al.* system was *Chlorella vulgaris* and the nutrient source was the same as in SBBR.

Biomass concentration in HBBR was the highest among all reference reactors and values reported by other scientists. The amount of dry biomass in HBBR comprised 25% of the total product weight and was equal to 345.3±68.6 g/L. Therefore, biomass obtained in HBBR can be used in various applications without any post-processing. High density of biomass eliminates the need for expensive dewatering step, significantly decreasing overall costs of production.

7.4.4 Water consumption

The estimated amount of water consumed by non-suspended reactors is given in Table 15.

Table 15 Water consumption of non-suspended reactors

Reactor	HBBR	SBBR
Water consumed per recommended growth cycle [L]	1.9	10.4
Amount of biomass produced during growth cycle [g]	0.06	0.12
Amount of water to produce g of biomass [L/g]	31.68	86.67
L of water required to support the growth in 1 dm^3 of reactor	0.02	1

The total amount of water consumed per growth cycle was the lowest in HBBR. This was mainly due to the fact that the reactor did not require any initial amounts of liquid. Water was supplied to the cells at 0.27 L per day as a mist using a fogger. The mist was not recycled, however, there is potential for it. Without recycle stream, the HBBR system consumed approximately 32 litres of water including medium vaporised by nebuliser. The consumption of water in SBBR was significantly higher (87 L). Increased water consumption was mainly caused by the high volume of initial water input. Further studies on the packing densities of HBBR and SBBR would enable the most accurate comparison of water consumption per g of produced biomass. The amount of water needed by

HBBR to produce one gram of biomass could be potentially lower for higher number of accommodated samples. Considering the volume of water used per volume of reactor, the consumption of water in HBBR was much lower than in SBBR. 0.02L of water was used to support growth in dm^3 of the HBBR system in comparison to 1 L of water required per dm^3 of reference non-suspended reactor. As mentioned before, packing density is a maximum number of substrates that can be placed in dm^3 of a given system. With known packing density, it is possible to evaluate the system's full potential in terms of productivity and water consumption. Packing densities of HBBR and SBBR are not known.

7.4.5 HBBR performance

As mentioned in the section 7.4.1, the highest amount of biomass was produced in SBBR (3.2±0.1 cells·10^6/cm^2). However, SBBR needed additional two weeks to reach maximum biofilm density compared to HBBR. *Chlorella saccharophila* in HBBR grew much faster than in any of the other reactors during exponential phase (0.15 compared to 0.07 and 0.07 day^{-1} in SBBR and SPBR, respectively). In HBBR, biofilm density reached 1.8±0.2 cells·10^6/cm^2 in 7 days. When considering 7 days cycle, the reactor will produce around 5.4 cells·10^6/cm^2 in three weeks, whereas at the same time, SBBR would produce only 3.2±0.1 cells·10^6/cm^2. Therefore, high initial growth in HBBR system would allow producing higher amount of biomass over a series of growth cycles.

To recycle water from SBBR and SPBR, water used as medium needs to be processed; some cells usually remain in post-treatment water. Recycled water containing microalgal cells cannot be applied in processes other than microalgal cultivation. In contrast, stream of recycled water from HBBR could be used for other applications, as water recovered from HBBR does not contain any microalgal cells. Moreover, recycle stream of steam can be led back to the reactor to feed the fogger and thus further cutting the water consumption in HBBR. Therefore, water utilisation in HBBR would be much more effective than in remaining reactors. Water after the process can be utilised in other process

associated within the same industrial plant or led back to the reactor, which would result in lower water consumption in comparison to reference reactors.

In addition, biomass produced by HBBR had much lower water content than remaining reactors. It means that the reactor does not require any additional post-processing, which significantly lowers overall costs of the process. In comparison with reference reactors, harvesting step in HBBR is much simpler and cost effective.

Table 16 HBBR and SBBR comparison

System	HBBR	SBBR
Highest growth rates [day^{-1}]	0.15	0.07
Lag phase duration [days]	0	7
Biomass concentration [g of dry biomass/ L of water]	345±69	97±32
Amount of water for post-treatment [L per 100g of product]	0.3	1.0
Medium	BBM	BBM
Medium delivery method	Mist	Liquid
Arrangement of samples	Horizontal	Horizontal
Issues	Oversaturation	Lag phase. Additional growth of algae cells outside biofilm

Comparison between HBBR and SBBR systems is given in Table 16. To sum up, HBBR seems an attractive solution for microalgal biomass cultivation, when compared to reference reactors. Growth of *C. saccharophila* in HBBR system was more rapid and higher biomass densities were obtained in shorter time period. Application of re-growth cycles would further increase the productivity. The biomass concentration of final product obtained in HBBR was much higher

than in remaining reactors and less water was required in the cultivation. Moreover, the maintenance of the system was easier, as all samples were easily accessible at any time of cultivation.

7.4.6 HBBR environmental limitations

HBBR system has certain environmental limitations. One of them is temperature build-up. Temperature oscillated between 25-35°C and it was not easy to control. Introduction of steam outlet helped in temperature build-up, although the reactor was still highly susceptible to temperature variations of the surrounding environment.

Relative humidity level inside the reactor was strongly correlated to already mentioned temperature and to the operation regime of the fogger. Temperature oscillations can cause humidity oscillations, which were not good for the biofilm inside HBBR. During oversaturation periods, the steam accumulated under the lid of the reactor and was falling down on the samples in the form of water droplets. Steam liquefaction contributed to washing off of some algal biofilms. So far, the fogger was operating at fixed time. Therefore, appropriate control of fogger could additionally improve humidity level inside the reactor, as the device could shut down during oversaturation periods and resume its operation when the humidity level inside the reactor is too low.

Another environmental limitation was light requirement. The reactor needs additional growth trials determining the most optimal light intensity. The light intensity used in the reactor was in accordance to CCAP guidance, although it is not known whether it is the most appropriate and effective value for the maximal biofilm growth,

The exact amount of nutrients that reach the surface of biofilm is not known. Nebuliser produced nutrient-rich steam which was distributed throughout the whole reactor. Growth trial investigating the effective amount of nutrients utilised by biofilm needs to be performed.

7.4.7 Comparison to other biofilm bioreactors and future work recommendations

HBBR performance was compared with other investigations on biofilm-based photobioreactors presented in Table 17.

The most promising biofilm-based systems proposed so far are rotating photobioreactors. The field test showed that the systems proposed by Christenson and Sims [32] and Blanken *et al.* [7] could reach up to 30 and 20.1 grams of biomass per m^2 per day, respectively. It is, however, important to distinguish between wet biomass productivity and dry weight biomass productivity. Some studies done on attached systems express the productivity in terms of final product weight [7] [21] [32] [22]. Nevertheless, the final product may contain a significant amount of water, so the actual amount of dry biomass produced inside the reactor is not known. *C. saccharophila'*s productivity was compared to those of other non-suspended solutions, some of the productivities were recalculated to allow comparison.

Table 17 Biofilm bioreactors compared

Method	Lee *et al.*, 2014	Schnurr *et al.*, 2013	Ozkan *et al.*, 2012	Genin *et al.*, 2014	Gross *et al.*, 2013	Johnson and Wen, 2010	Christenson and Sims, 2012	Blanken *et al.*, 2014
Reference	[18]	[19]	[20]	[34]	[21]	[22]	[32]	[7]
Purpose	biodiesel production	nutrient starvation experiment	energy and water requirement decrease	material surface energy effects on algal film productivity	development of widely adopted system for easy biomass harvest	biofuel production	biofuels production, wastewater treatment	microalgae biomass production
Advantages	simpler dewatering and harvesting	higher productivity than crops	Significant reduction of the energy and water requirements	-	easy biomass harvest, enhanced biomass productivity	easy and less expensive than suspended cultivation	greater biomass production, FAME production, nutrient removal, efficient energy balance	High biomass productivity, scale-up possibility
Biomass productivity [g/m^2/day]	9.1	no data	no data	no data	3.51 (closed), 12.76 (open)	40.8	5.5-31	20.1
Mass fraction biomass to water [g of biomass/L of water]	no data	no data	no data	no data	97	63	120-160	143-192
Dry weight biomass productivity [g of dry product/m^2/day]	no data	2.8 (*N.palea*), 2.1 (*S.obliquus*)	0.71	1.10-2.08	0.34 (closed), 1.24 (open)	2.57	0.66-4.96	2.87-3.86
Reactor arrangement	vertical	horizontal	titled at 0.2	vertical	traingular and vertical	rocking motion at 15° from the horizontal plane	rotation	vertical, rotation
Reactor type	open (raceway	closed (PBR)	closed (PBR)	closed	closed (RAB) and open (pond with	closed	closed (lab scale), open	closed

	pond)				RAB)		(pond)	
Microalgal strains	*Scenedesmus, Chlorella, Pediastrum, Nitzschia, Cosmarium*	*Scenedesmus obliquus, Nitzschia palea*	*Botryococcus braunii*	*S. obliquus, C. vulgaris, Coccomyxa* sp., *Nannochloris* sp., *Nitschia palea, Oocystis* sp., *Occystis polymorpha*	*C. vulgaris*	*Chlorella* sp.	*Chlorella, Scenedesmus, Pediastrum, Nitzschia, Navicula, Crucigenia, Syneddra, Diatoma*	*C. sorokiniana*
Materials	polycarbonate plate, polyethylene plate, **nylon mesh**, stainless mesh	glass	concrete slab	acrylic, glass, polycarbonate, polystyrene, **cellulose acetate**	muslin cheesecloth, fiberglass (armid, PTFE coated), chamois leather cloth, vermiculite, microfiber, synthetic chamois cloth, burlap, **cotton duct**, velvet, tyvek, poly-lactic acid, nylon, polyester	**polystyrene foam**, cardboard, polyethylene landscape fabric, loofah sponge, polyurethane foam, nylon sponge	nylon, polypropylene, cotton, acrylic, jute, polyester, high thread cotton, low thread cotton	stainless steel woven meshes, sanded polycarbonate disk
Medium	wastewater	wastewater, medium with 10% wastewater, 2% CO_2 supply	BG-11	unsterile wastewater + FBBM (1:2), 1% CO_2 supply	BBM, CO_2 supply	dairy manure wastewater	wastewater	M-8 media, urea, $NaHCO_3$, CO_2 supply
Light source	Sunlight	8W red-light emitting diodes	32W fluorescent lamps	8W LEDs	artificial light source (closed), sunlight (open)	cool white fluorescent lights	plant growth fluorescent lights	warm-white directional LED light source
Reactor volume [L] or Cultivation area [m^2]	8000 L	0.072 m^2	0.275 m^2	15 L	0.045 m^2 (closed)	200 mL/ 0.0136 m^2	8-8000 L	21 L
Light intensity [umol/m^2/s]	272-520	80	55	no data	110-120 (closed)	110-120	290	422
Duration of experiment [days]	18	20-21	35	10	15	10	26	147 (7-days harvest cycle)
Removal method	scraping	scraping	scraping	scraping	scraping	scraping	scrapper blade harvester	scraping

HBBR productivity was the lowest among presented reactors (0.20 g/m^2/day). Low productivities inside the reactor highlight the necessity for further optimisation of the system. The most important issue to be addressed is the humidity inside the reactor. Maintaining its proper balance was one of the factors that influenced microalgal growth. Saturation led to microalgal biofilm being washed off, while lower humidity inhibited microalgal growth. Better humidity control (preferably automatic) would ensure steady growth of microalgae. Furthermore, the light intensity (42 µmol/m^2/s) inside the reactor could be too low for the biofilm. Compared to other work done on biofilm bioreactors, the light intensity applied in this experiment was very low. Although it was the value recommended by microalgal supplier, the case of biofilm bioreactors may require higher light intensities in order to be feasible.

A high number of systems combined microalgal cultivation with simultaneous wastewater nutrient remediation. It is a good approach, as the systems benefit from additional application. However, the contamination issue need to be raised. It is not easy to maintain the reactor to be microbes-free. The problem of contamination is particularly essential in pharmaceutical or nutraceutical applications of end product. It also disturbs the balance, especially in case of biofilm consisting of axenic microalgal culture. HBBR system showed good level of contamination control. During 42-days of growth trial no presence of other microorganisms was observed. Additional benefit of HBBR is that the system limits microalgae contact with external environment.

Vast majority of biofilm-based cultivation systems rely on scraping as a harvesting method. Simple scraper can be used and costs associated with harvesting are very low for all systems. Further post-processing strongly depends on the application of final product. For some applications, like fish feed supplementing, high content of water (around 95%) is still acceptable. For other, such as biodiesel production, lower content of water is desirable, preferable no water should be present. From *Chlorella pyrenoidosa* biomass of 0% water content, it is possible to obtain 91.4% biodiesel yield [407]. When the water concentration increases to 90%, the biodiesel yield significantly drops to 10.3%

[407]. Additional dewatering system may be required for low biomass concentrations. Dewatering is an expensive process and may significantly increase the cost of the whole system. HBBR is characterised by exceptionally high biomass content, up to 25%. Similar biomass content is obtained after dewatering in suspended cultivation methods [42]. Biomass concentration in HBBR was the highest among reported values and was equal to 345.3±68.6 g/L. Biomass content of end product from HBBR mostly depends upon optimisation of the reactor and is the highest when accurate humidity level is maintained, preferably close to 100% RH. Therefore, mechanisation and humidity control inside the reactor are important. Optimised HBBR can meet biomass content requirements of any industry and does not need any additional post-processing except cheap scraping.

Various materials were used as attachment substrates. The most important parameters which need to be considered when applying the material in biofilm-based systems are: durability of the material, its low price and low absorptivity. Durability of the material is important, as subsequent scraping may damage the material. The best material would be able to withstand high number of re-growth cycles. In addition, good attachment material should not absorb water. It is more difficult to scrap biofilm from absorptive surface, as part of microalgae cells grow inside the substrate, not on its surface. Such phenomenon was observed in experiments with cotton. Plastics seem to be the best solution for biofilm-based cultivation approach, which is reflected by highest biomass productivities on those materials among other types of substrates.

Position of material can be horizontal, vertical or sloped. HBBR reactor design allows any arrangement of samples inside the reactor. Flexibility in the HBBR sample arrangement facilitates scale-up of the system. So far, the simple design of HBBR includes single row of samples. HBBR can be scaled-up by stocking additional substrate surfaces in rows. Substrates can be also aligned vertically and placed in columns.

Biofilm-based cultivation approach is a rather new solution for microalgae cultivation. That is why most of the systems compared above are at laboratory

scale stage. It means that they need to utilise artificial light and artificial CO_2 supply, which generate additional costs. Moreover, to commercialise biofilm-based systems, it is essential to decrease energy consumption to minimum. The common approach is utilisation of laboratory scale reactors as open systems. In open systems, microalgae have an access to natural sunlight and CO_2 from the atmosphere, which decreases the costs significantly. However, microalgal culture may be prone to contaminations and system maintenance is difficult, mainly due to vast amount of water present. HBBR is an attractive alternative to already existing open ponds, as the system can be tested at external environment. The characteristics of UK climate may allow the system to benefit from high humidity and low temperature. Placing system in the greenhouse would ensure contamination control, while low temperature will prevent overheating inside greenhouse. Relative humidity levels in some UK cities can reach up to 90% [411], which will decrease generation of artificial humidity to minimum.

HBBR is not optimised yet, and it still requires additional work. It is essential to automatize the humidity control in order to avoid unexpected saturation or drying. Adding mist recycle stream will allow reusing the steam that escapes the reactor. So far, the steam was permitted to leave the chamber in order to decrease the risk of oversaturation. Both improvements would result in efficient steam production control and they would further decrease the water consumption.

Test trials inside the reactor need to be performed for a longer period of time with one week growth cycles (ended by scraping and subsequent re-growth). As it was showed by Blanken *et al.* [7], re-growth cycles are characterised by higher biofilm growth than start-up cycle. Re-growth approach can increase the overall productivity of the system.

7.5 Conclusions

Novel cultivation method was proven to be an attractive alternative to the cultivation techniques currently used. The benefits of the new method include

lower water consumption, easier maintenance of the system and easier scale-up of the system.

The biomass density inside the reactor reached $1.9\pm0.1\cdot10^6$ cells/cm^2 at exponential growth phase, which was the highest in comparison to reference reactors studied in this chapter. The biomass concentration reached up to 345 g/L eliminating the need for biomass post-processing.

The suggested re-growth cycle duration was 7 days, which was three times shorter than that of the reference reactors. Further optimisation of the novel cultivation system is necessary in order to enhance production yields and reactor performance. Moreover, longer duration of experiments and re-growth cycles are required.

8 Overall implications for biofilm growth in humid environment

8.1 Mechanisms controlling algal biofilm formation

The formation of microalgal biofilm is mostly governed by physico-chemical interactions between cells, medium and attachment substrate. When the negatively charged microalgal cells approach the surface of attachment they need to overcome the electrostatic repulsive forces in order to adhere to it. These interactions and the factors impacting on microbial colonisation are still relatively poorly understood. This thesis set out to produce a new aeroterrestrial biofilm-based bioreactor and in doing so investigated the role of material and microalgal properties on biofilm initiation and development. Most of the work done on this subject has been focusing on limiting the growth of fouling biofilm whereas the aim of this work was to aid their establishment and development.

8.1.1 Substrate of attachment

The topographical characteristics of the material play a significant role in microalgal species colonisation as it can inhibit or enhance adhesion, depending on the size and arrangement of structural features [12]. Microalgal strains tend to adhere more to substrates that provide shelter against shear forces of moving liquid. The presence of cavities and elevations has a strong impact on the quality and quantity of the biofilm formed. In principle, the higher the number of cells attached the higher the amount of biofilm produced.

This work showed that on rough substrates the cells needed to adapt their deposition according to the structural characteristics of the materials, forming shape and patterns that overlapped material topography. On smooth materials microalgal cells adhered randomly but they were then able to form uniform and well-organised matrix of cells. Cavities of bead blasted steels and elevations of polystyrene helped microalgal attachment by increasing their resistance to shear forces of moving liquid. It is therefore possible to engineer appropriate surface topography that would enhance quantity and quality of the initial attachment.

Physico-chemical properties of the materials are also very important for primary colonisation and they are usually exploited to reduce biofilm formation (for example in marine biofouling or medical devices). Chemical compounds that constitute the material can interact with the microalgal cells and reduce or enhance their attachment. Furthermore, this work showed that the elastic modulus of the materials influenced the cell release from the substrate and contributed to enhance the detachment rate of reversibly adhered cells. Materials with higher elastic modulus, such as polystyrene or polyethylene terephthalate, also promoted primary colonisation of tested microalgal strains.

8.1.2 Microalgal strain

The quantity and quality of the formed biofilm is strongly species-dependent. Some species naturally grow in biofilm communities and it is easier for them to switch from planktonic to sessile state; whereas other species prefer to grow in suspensions.

The polarity of microalgal strains played the most significant role in their attachment. Cells with higher polarity had to overcome higher repulsive forces during attachment, therefore it was difficult for them to colonise the substrates.

In addition, the ability of microalgal species to secrete algogenic organic matter (AOM), composed of soluble microbial products (SMP) and extracellular polymeric substances (EPS) enhances biofilm formation. Both SMP and EPS help in the earliest steps of cell attachment. An initial conditioning layer composed of SMP promotes microalgal adhesion. Once attached the cells start releasing EPS which help connecting cells and forming chains which will provide the structure of the biofilm matrix. Aggregates of microalgal cells are therefore embedded in EPS that strengthen the biofilm structure and allow a more efficient transport of nutrients.

8.2 Mechanisms controlling algal biofilm development in liquid and humid environment

Biofilm community protects cells against stress conditions in both liquid and humid surroundings. However, the mechanism of biofilm development is slightly

different in the two environments first and foremost because in humid atmosphere water availability is the critical factor for cell multiplication. The main mechanisms controlling biofilm development are biological interactions, such as cell-to-cell communication or secretion of organic and inorganic compounds. From that perspective, the biofilm quality is crucial for efficient cells growth and enhanced nutrients transport. In addition, interactions between algae and bacteria influence biofilm formulation and development. There are different types of association between microalgae and bacteria. In mutualism, both microorganisms benefit: for an example, when bacteria provide vitamin B_{12} in exchange for fixed carbon [412]. In commensalism, only one partner benefits: an example is lower growth of phosphate-limited microalgae that allow bacteria to outnumber them [413]. Parasitism is the most well studied interaction between bacteria and microalgae: in this association, one of partners benefits, while the other is negatively affected. An example of such interaction is microalgal cell degradation caused by bacteria present in microalgal cell wall [414]. Algal-bacterial biofilm systems are gaining an interest, as bacteria can improve biomass production rates of some microalgae. Hoverer, such systems still require further investigation [415]. In this study, the presence of bacteria was evaluated only under the microscope; therefore it is possible that association between bacteria and microalgae took place. Further test would need to be carried out in order to confirm or deny bacterial presence.

8.2.1 Substrate of attachment

Properties of materials, such as microtopography, can have a direct impact on biofilm growth, as they influence microalgal development by promoting higher quality of adhesion. Surfaces that lack of any structural features, such as cavities or elevations, allow cells to attach in a random way but then develop in a more organised manner. Well-structured biofilm results in better nutrient transportation between cells and promote cell-to-cell interactions. Even though smooth materials do not provide shelter against shear forces of moving liquid, EPS embed cells together and act as a protective agent against environmental conditions. On the other hand, cavities and elevations of rough substrates can

limit further development of microalgal biofilms as cells do not have space to multiply. Nutrient transportation and AOM secretion are limited, as more cell-to-substratum interactions and less cell-to-cell communication take place. Data from this thesis showed that growth rates of microalgae were higher on smoother polyethylene terephthalate and sand polished steels, than on their rougher equivalents in both humid and liquid atmosphere.

8.2.2 Microalgal strain

In the liquid environment, microalgae were secreting increased amounts of AOM, to help biofilm formation and development. As described earlier, EPS influenced the strength and physical properties of mature biofilm and also helped in self-induced detachment at the end of biofilm growth cycle. EPS degradation allowed cells to escape the nutrient-depleted biofilm in search for new nutrient sources. Moreover, decreased levels of EPS change biofilm polarity, potentially preventing the co-aggregation of new species. The ability to secrete AOM and its composition are therefore crucial for choosing the right algae for biofilm-based growth.

In the humid environment the four species tested showed different behaviour. The humid environment is much more challenging the liquid one, and causes more stress to the cells. One potential explanation for the different behaviour is the cells ability to produce stress metabolites such as polyols. These metabolites were proven to influence the ability of microalgae to withstand environmental stress conditions. In our study *Stichococcus* sp. that was not able to metabolise polyols, had significant problems maintaining its growth in the humid reactor. On the contrary, microalgae that can metabolise ribitol were growing well in the same conditions. The polyols production was not tested; hence this hypothesis will need to be proved.

8.3 Potential for implementation

A novel cultivation reactor was designed to address some of the sustainability issues associated with traditional cultivation techniques and to efficiently produce microalgal biomass at lower cost. Expected commercial benefits of this

project were: lower water consumption, faster biomass growth, and cheaper or no harvesting step.

The novel technology proposed in this thesis could have a potential use on a full-scale operating system. The humid biofilm bioreactor (HBBR) was designed to be easy to scale-up due to the modularity of its structure. The number of samples inside the reactor could be easily increased by optimising the rows distance. In addition, the low costs of the reactor's parts and simple working principles of the system would enable construction at large scale. Further reduction of the costs associated with scaled-up system could be achieved using flashing LED lights inside the growth plates or naturally humid environments.

In this thesis, the HBBR was validated at laboratory scale (phase 1-4, Table 18). HBBR was proven to effectively produce microalgal biomass with lower water consumption in comparison to other reference reactors. The concentration of biomass obtained in HBBR was the highest among all biofilm-based systems reported so far.

Table 18 Project phases

Phase	Level	Technology Readiness
Basic technology research	1	Basic principles observed and reported
	2	Technology concept formulated
Research to prove feasibility	3	Proof of concept of key analytical characteristics
Technology development	4	Laboratory validation of technology
	5	Pilot scale validation of technology
Technology demonstration	6	Prototype demonstration and process optimisation

System development	7	Prototype demonstration in an operational environment
System test, deployment and operations	8	Full scale system completed and tested
	9	System proven in successful end use operation

Validation at pilot scale, prototype demonstration and process optimisation are still required for implementing the system for end-use operation. When proved to efficiently operate at pilot scale, the system could be the most effective approach to produce microalgal biomass amongst already existing methods.

9 Conclusions and future work recommendations

This thesis addressed certain research gaps in the field of biofilm deposition and development. Following conclusions were drawn in regard to the main thesis objectives:

Objective 1. *To produce a state of the art review on aerial-non-suspended microalgal growth*.

- Review showed that there is an emerging need for a new, more efficient microalgae cultivation approach.
- Water consumption and its associated costs for separation and extraction are the key steps to address for increasing the environmental sustainability of microalgae processes and lowering their final production costs.
- With higher yields, lower water consumption and low-energy harvesting costs, biofilm bioreactors can address the environmental impact of microalgae processes. Energy consumption for lighting is also reduced in these systems because of the improved light distribution, not limited by culture density.
- Post-processing costs in biofilm bioreactors are lower than those of suspended cultures.
- With minimum water requirements (humidity), enhanced light penetration and distribution and easy maintenance, aerial biofilm cultivation has the potential to further decrease the environmental impact of microalgal cultivation.
- New cultivation approach proposed in this thesis is the most promising alternative to already existing methods.

Objective 2 and 5. *To evaluate and compare primary attachment of aeroterrestrial and freshwater strains on different materials*. *To estimate non-aqueous biofilm-based growth of different microalgae species.*

- Cell attachment and biofilm growth was species-dependent, although it was not dependent upon isolation environment (aeroterrestrial/freshwater microalgae).
- Aeroterrestrial *Stichococcus* sp. cell was secreting more EPS than freshwater *C. saccharophila* cell, which meant that *Stichococcus* due to lack of polyols required higher concentration of EPS in response to stress conditions.
- Both aeroterrestrial and freshwater microalgae can be effectively grown in humid and liquid environment.
- Aeroterrestrial *C. luteoviridis* was as good candidate to be grown in novel biofilm bioreactor as freshwater *C. saccharophila*; the final choice of strain had to be made in regard to their growth on selected attachment material.

Objective 3. *To identify and appraise the factors affecting primary adhesion and biofilm development*.

- The polarity value of individual algae strain impacts the primary attachment onto surfaces. The primary attachment was lower for strains of higher polarity as more polar microalgae have to overcome higher repulsive forces to adhere onto substrates.
- Surface roughness has not got influence on primary adhesion; it was rather the substrate structure and characteristic features that promoted well-structured adhesion of cells (smooth materials) or provided a shelter from liquid shear forces inside cavities (rough substrates).
- Primary adhesion is promoted on substrates with attachment points; however, further growth is better on materials that did not possess characteristic structural features.
- Material structure has an influence on biofilm development and algal organic matter (AOM) secretion. In addition, SMP and EPS concentration have an influence on strength of mature biofilm.
- EPS has an influence on biofilm polarity, the increase in EPS concentration is coupled with decrease in biofilm polarity. Hence, when polarity increases the biofilm is reaching sloughing stage, at which auto-detachment and enzymes-triggered breakage of EPS takes place.

Objective 4 and 6. *To design and develop a novel non-aqueous biofilm-based growth system. To optimise the non-aqueous biofilm-based reactor*

- Concept of aerial cultivation for biomass production was proved to work for most microalgal species and materials.
- HBBR is an attractive alternative to already existing microalgal cultivation methods, due to low water consumption, efficient light distribution, high biomass concentration, higher growth rates and easy maintenance.
- HBBR system allows producing microalgal biomass with high biomass content, the highest than values reported by other scientists.

9.1 Future work

Future work recommendations mainly comprise suggestions for HBBR improvement and more detailed investigations on its working principles.

9.1.1 Inoculation stage optimisation

Inoculation is an essential first step of biofilm cultivation in HBBR system. Due to the fact that the reactor is not situated in liquid environment, initial biofilm formed on adhesion carrier is the only biomass available at the beginning of the cultivation; no co-aggregation can take place in HBBR reactor. Therefore, inoculation stage should result in the highest quality and quantity of biofilm.

Future work recommendations for inoculation step include additional trials determining the colonisation ratio in relation to inoculation stage duration and initial density of inoculum. Shorter and longer duration of inoculation should be tested, so that the optimal time of contact between inoculum and attachment carrier could be identified. It was also observed that there is a certain maximum amount of cells that can adhere into the substrate surface during an inoculation stage. At some point there is no increase in colonisation of cells for increasing inoculation density. The most optimum initial inoculum density needs to be identified.

9.1.2 HBBR optimisation

Measurements on the amount of nutrients that reach each sample would need to be conducted in order to evaluate the recommended concentration of medium. Moreover, additional growth trials are needed. They would determine what concentration of nutrients and frequency of dosing are resulting in the most efficient growth rates and the highest biofilm density.

A full growth cycle that will comprise of several one-week re-growth cycles need to be conducted. The study would show how much biomass can be produced in cyclic cultivation. It will also suggest how well cells grow in cycles following the initial growth cycle and whether lag phase takes place.

The novel biofilm bioreactor requires further optimisation. Two separate vessels would ensure better humidity control inside the reactor. The fogger (Figure 48 A) should be placed in a separate tank, so that no water droplets can form inside the reactor. Even distribution of humidity would be ensured by a fan (Figure 48 B), also placed in the first vessel. This solution would increase the number of samples inside the reactor vessel; samples could be placed lower without the risk of biofilm being washed off by the fogger droplets. Nebuliser inlet would be connected into steam inlet (Figure 48 C), so that the steam entering the main reactor chamber would already contain nutrients. Liquefied water would be collected from the bottom of the reactor and fed to the mist chamber (Figure 48 D). Mist excess would be collected from the top of the reactor and fed back to the steam chamber (Figure 48 E).

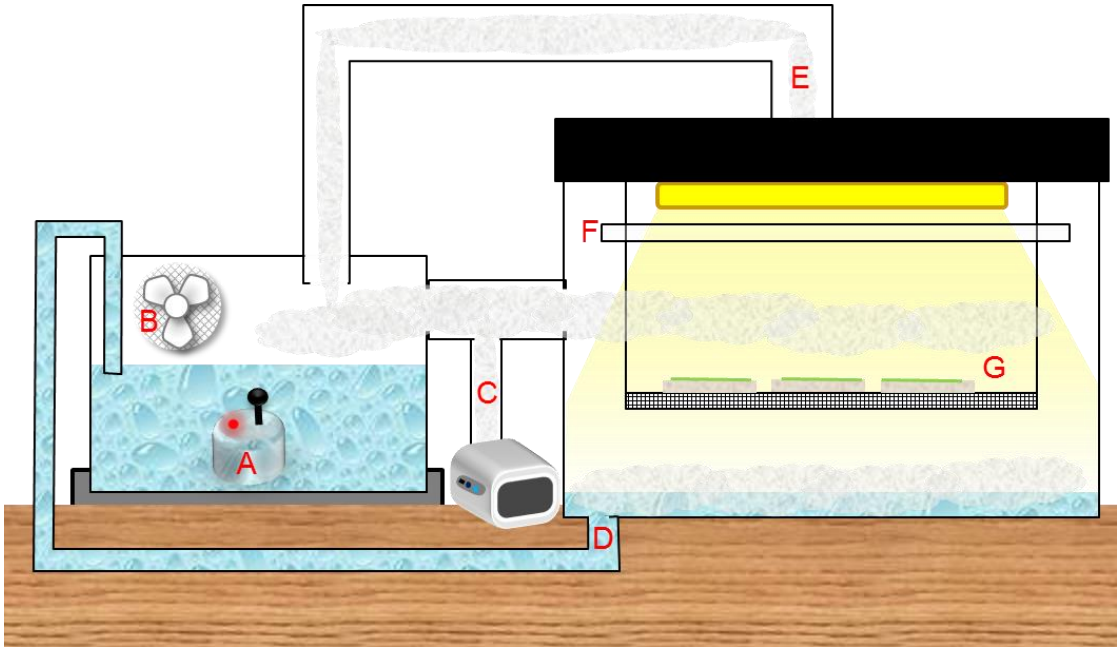


Figure 48 Suggested design of upgraded HBBR

Accurate humidity control would eliminate or reduce the problem of oversaturation. If oversaturation still occurs, engraved slope (Figure 48 F) is suggested to be placed above samples (Figure 48 G). Water forming at the lid of the reactor would be directed into side walls and then to the bottom of the reactor with the help of engraved slope.

Scale-up of the bioreactor would give an insight into potential packing density of the system. It is suggested to increase the number of samples rows in order to accommodate higher number of samples. The most optimum distribution of samples would need to be investigated in a series of experiments.

9.1.3 Industrial/commercial investigation

One kilogram of microalgal biomass commercially costs approximately $2 when produced in open ponds and approximately $13 when produced in closed systems [416]. Using wastewater as a nutrient source and utilising CO_2 in the cultivation the process can decrease those costs even further. However, the vast majority of costs are still linked to the dewatering step. Researchers propose various designs of cultivation systems that result in a more efficient biomass production [417] [418]. However, it is not possible to achieve low costs

of microalgal biomass solely by increasing production yields. Removal or reduction of the post-processing step could significantly lower the costs of microalgal production and improve the feasibility of the overall process. Systems like HBBR, with lower water consumption and higher concentration of end product are a potential solution to the problem. The cost of biomass produced in laboratory scale HBBR system was estimated to be significantly higher than the cost of producing one kilogram of microalgal biomass in commercially operating photobioreactors. However, when compared with growth of the same strain at similar conditions in incubator shaker Innova®44, the costs are ten times lower. The difference arises mostly from additional post processing (incubator shaker) and low initial costs of HBBR system.

	HBBR	**HBBR (10 units)**	**Innova®44**
Initial costs	£450	£2,500	£20,000
Consumables per year	£70	£700	£600
Biomass produced per year [kg]	0.15	1.5	0.6
Cost per g of biomass	£3	£2	£12

Figure 49 Cost estimation comparison between HBBR and Innova®44 photobioreactor

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APPENDICES

Appendix A Materials selection

Short growth trials at humid atmosphere (described in Chapter 4) were used to identify the most appropriate strain and material that would be used inside the reactor for longer trial. Various aspects needed to be considered in the selection process. The material needs to be cheap and durable to withstand as many re-growth cycles as possible [419]. Primary attachment and growth rates on the material should be as high as possible and harvesting by scraping should be easy. The properties of materials tested in chapter 3, in regard to these requirements are summarised in Table 19. For potential large scale applications the cheapest materials were plastics: PETG, PP and PS. Microalgae preferred to adhere to polystyrene. Even though the growth rates were not very high, the final number of cells in 5-days old biofilm was satisfactory (4.6±0.2 cells·10^6/cm^2 for *C. luteoviridis* and 5.9±0.2 cells·10^6/cm^2 for *C. saccharophila*). Other materials with overall high initial attachment were PETG, sand polished steel 316 and bead blasted steels 316 and 304. The remaining materials were moderately good carrier substrates; only glass was not satisfactory with the lowest primary adhesion equal to 4.5±1 cells·10^5/cm^2 for *H. pluvialis*.

Table 19 Comparison of materials

Material	Price per m^2 *	Initial colonisation, ρ [cells·10^6/cm^2]	Growth rates, r	Durability for re-growth cycles	Scraping	Biofilm layer after scraping	Resistance to oversaturation
stainless steel grade 316 bead blasted (SS 316 BB)	£198.83 [420] + blasting	1.6-3.3	0-0.24	High	Difficult	Biofilm layer left	Moderate
stainless steel grade 316 sand polished (SS 316 SP)	£198.83 [420]	1.1-3.8	0.22-0.26	High	Moderate	Small	Low
stainless steel grade 304 bead blasted (SS 304 BB)	£198.83 [420] + blasting	1.5-2.9	0-0.33	High	Difficult	Biofilm layer left	Moderate
stainless steel grade 304 sand polished (SS 304 SP)	£198.83 [420]	8.9-3.0	0.13-0.27	High	Moderate	Small	Low
Polypropylene (PP)	£12.33 [421]	1.0-2.1	not tested	High	Easy	None	Low
Polyetheylene terephthalate glycol modified (PETG)	£14.58 [421]	1.7-2.4	0-0.19	High	Easy	None	Low
polyvinyl chloride (PVC)	£24.89 [421]	1.4-2.4	not tested	High	Easy	Small	Low
polystyrene (PS)	£19.30 [422]	2.9-3.3	0-0.17	High	Moderate	Biofilm layer left	High
glass	£22.50 [423]	0.4-1.3	High	High	Easy	None	Low

* Cost of individual materials may vary amongst suppliers

Moderate primary attachment on sand polished steels was compensated by very high growth rates. SS 304 SP and SS 316 SP had the highest number of cells in biofilm after 5-days cultivation (8.8±0.3 cells·10^6/cm^2 for *C. luteoviridis* and 10.7±1.5 cells·10^6/cm^2 for *C. saccharophila*, respectively). It was also surprising that cell division on glass was the fastest among all materials, with 0.40 day^{-1} growth rate for *Stichococcus* sp. However, the small number of initially adhered cells corresponded to low final density of the biofilm; only 1.6±0.1 cells·10^6/cm^2 remained attached at the end of cultivation time.

All materials withstand repeatable re-growth cycles. They were continuously applied in 5-days short growth trials, where their resistance to scraping and sonication was tested. No significant changes in the structure were observed during experimental investigations. Moreover, no tear or wear of materials presented above took place as it was observed in the case of cotton and parafilm substrates . Nevertheless, biofilm harvesting was performed with varying efficiency; only a part of microalgae could be removed from some materials, while on the others the whole biofilm could be detached by scraping. This is especially important for re-growth cycles as it is necessary for some cells to remain on the substrate for re-entering another growth cycle. From that perspective, PS and bead blasted steels were the best materials. Even though it was not easy to remove the biofilm from a rough substrate, a considerable amount of cells was left for further re-growth. As it was proven by Blanken *et al.* [7], the re-growth cycle of microalgae is more efficient than the initial growth.

Material's resistance to oversaturation is another aspect that play important role in substrate selection for HBBR system. The best material should be able to support the growth of microalgae when humidity level increases significantly. As mentioned in previous chapters, smooth materials promote further development of biofilm as shear forces do not influence the EPS-embedded biofilm. However, the specific shear forces in HBBR reactor are higher than movements of standing liquid. During local oversaturation, steam accumulated at the top of the reactor and liquefied. Liquefied water was falling down on the substrates in the form of droplets. Resulting water droplets moved not only because of vibrations,

but also due to gravitational forces. Therefore, droplets exerted forces that had high impact on developing biofilm. Appropriate material needs to be able to withstand oversaturation periods, as they are still an issue in the reactor. Among all tested the materials, only bead blasted steel and polystyrene indicated increased resistance to this phenomenon. Biofilm exposed to water droplets on these substrates was not washed off, as the structured surface of these materials prevented detachment.

Taking everything into account, polystyrene was selected as the preferred substrate material due to its low price, high durability, high resistance to oversaturation and very high initial attachment coupled with satisfactory growth rates. Moreover, PS structure allowed a biofilm layer to remain on substrate after scraping, which is essential for re-growth cycle.

Appendix B Microalgal strains selection

Microalgal strain was selected based on initial adhesion, growth rates and final biofilm density on plastics. Selection criteria of microalgal strains tested in this study are summarised in Table 20.

All strains had comparable multiplication times. In average, microalgae needed from 2 to 5 days to double their cell density. *C. saccharophila* had the most efficient doubling time equal to 2.59±1.13 hours, while *Stichococcus* sp. growth was the slowest among tested strains with doubling time equal to 4.57±1.64 hours.

Initial colonisation on plastics was high for all microalgal strains, in particular for *Stichococcus* sp., *C. saccharophila* and *H. pluvialis*. The primary attachment on selected PS was very similar among strains, with an average 3.1±0.2 cells·10^6/cm^2. However, there was a significant difference between strains in the further growth on this material. *Stichococcus* sp. had declined growth on PS and only 9.2±2.7 cells·10^5/cm^2 remained after 5-day cultivation. The highest growth rates were obtained by *H. pluvialis* and *C. saccharophila*, with the latter achieving the highest amount of cells in developed biofilm, equal to 5.9±0.2 cells·10^6/cm^2.

Table 20 Comparison of microalgal strains

Microalgal strain	Price [per strain]	Average doubling time [h]	Initial colonisation on plastics, ρ_i [cells·10^6/cm^2]	Growth rates on PS, r	Final number of cells on PS after 5 days of cultivation, ρ [cells·10^6/cm^2]	Applications
H. pluvialis	£50 [424]	3.78±1.56	1.7-2.9	0.09	4.2±0.2	astaxanthin [2]
Stichococcus sp.	£50 [424]	4.57±1.64	1.6-3.3	0	0.9±0.3	bioremediation [188]
C. luteoviridis	£50 [424]	3.68±1.21	1.0-3.3	0.08	4.6±0.2	wastewater treatment [189]
C. saccharophila	£50 [424]	2.59±1.13	1.5-3.0	0.17	5.9±0.2	lipids [192]

Limited research on *Stichococcus* sp. and *C. luteoviridis* was mostly focused on their accumulation on building facades and resistance to different stress conditions [80] [83]. Karapatsia *et al.* [426] study on *Stichococcus* sp. gave the mass fraction of bio-chemicals inside this microalga. It was found that *Stichococcus sp*. consists of 40.6% carbohydrates, 26.3% proteins and only 11.6% lipids by dry weight. Therefore, due to too low lipid content, it is not possible to use *Stichococcus sp*. in biofuel production by directly processing extracted lipids. Similar observation was made in the case of *C. luteoviridis*; the amount of lipids constituted around 8% of its biomass weight [189]. Both aeroterrestrial microalgae tested in this study are not rich in lipids, although they can still be used for other industrial applications.

C. saccharophila was proven to be a good biodiesel source. It was able to produce 63.3 mg/l/day and up to 99.3 mg/l/day under stress conditions [192], which accounted for 41% and 62% of dry biomass weight, respectively. Other studies also confirmed high lipid concentrations in *C. saccharophila*.

H. pluvialis is a well-known source of astaxanthin. Astaxanthin has many applications in nutraceutical and pharmaceutical industries due to high antioxidant properties [425]. Astaxanthin secretion by *H. pluvialis* is enhanced in

stress conditions, therefore the production system need to comprise of two stages: growth stage and astaxanthin production stage. Attached cultivation was proven to be an efficient solution for beta-carotene production.

Based on Table 20, *C. saccharophila* was selected as the most appropriate strain to be grown in HBBR. Those microalgae showed high growth rates and the highest biofilm density on selected polystyrene. Moreover, it was widely studied by other researchers and can be applied as a biofuel feedstock.

Appendix C Methods and apparatus

C.1 Attachment density calculations

After overnight inoculation, two samples of each material are taken out from the container. Samples are sonicated inside water buffer, in order to detach cells from the material. Water buffer with cells is cell counted under Olympus CX41 microscope (Figure 50). When total amount of cells which attach on the substrate is known, it is possible to calculate attachment density (ρ). The remaining samples are placed inside the reactor for a certain time (t- time of growth). Growth at t=48h and t=76h is measured in the same manner as for inoculation (t=0).

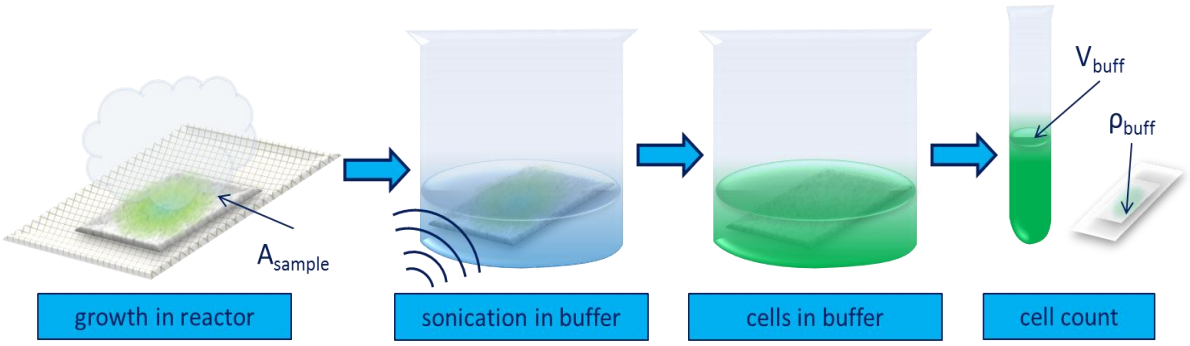

Figure 50 Scheme of growth density measurement

Volume of buffer and cell density is measured. Cell counting method is used to obtain amount of cells per ml of buffer. Multiplication by buffer volume gives the total amount of cells which were attached on the substrate. Dividing the total amount of cells by sample area gives the final cell density in cells per cm^2 (Equation 33).

$$\rho = \frac{\rho_{buff} \cdot V_{buff}}{A_{sample}}$$

Equation 33 Final cell density

where

ρ- attachment density/ growth density [cells/cm^2]

C.2 Biofilm growth

To compare the growth inside novel attachment cultivation reactor and suspended reactor, growth rate was introduced. It is a parameter which describes how fast microalgal cells are multiplying. Growth rate is an intensive quantity which does not rely on the exact amount of cells attached, but on the difference between initial and final amount of cells (Equation 34). That is why usage of growth rate is convenient when comparing systems of different densities.

$$r = \frac{\ln(\frac{\rho}{\rho_0})}{t - t_0}$$

Equation 34 Growth rate

where

r- growth rate [day^{-1}]

ρ_0- attachment density at t=0

t- growth time [days]

t_0- initial time (equal to 0)

Appendix D Tables

D.1 Chapter 3: Primary attachment of microalgae strains

Table 21 Primary attachment of *H.pluvialis*, *C. saccharophila, Stichococcus* sp. and *C. luteoviridis* on 9 different substrates

		Primary attachment [cells·10^4/cm^2]			
No	Material	*H. pluvialis*	*C. saccharophila*	*Stichococcus* sp.	*C. luteoviridis*
1	SS 316 BB	271±7	285±10	160±16	328±103
2	SS 316 SP	242±17	380±14	110±13	203±52
3	SS 304 BB	157±13	250±15	154±12	294±33
4	SS 304 SP	106±17	137±12	86±8	300±27
5	glass	45±10	48±7	65±17	126±38
6	PVC	195±15	149±11	242±11	140±24
7	PETG	171±14	244±14	181±29	210±19
8	PP	181±13	210±10	161±12	101±12
9	PS	293±40	304±12	326±37	333±139

D.2 Chapter 3: Measurement of surface charge

Surface charges of *H. pluvialis, Stichococcus* sp., *C. luteoviridis* and *C. saccharophila* microalgal cells were measured with Malvern Zetasizer Nano Z and are presented in Table 22. Zeta potential and electrophoretic mobility of cells was measured in Bold Basal medium aqueous dispersion at given pH values. From the results presented below, it can be assumed that all cells were charged negatively.

Table 22 Zeta potential and electrophoretic mobility of microalgal cells

Microalgal strain	Zeta potential (mV)	Mobility (µmcm/Vs)	Conductivity (mS/cm)	pH
H. pluvialis	-11.4±0.4	-0.89±0.03	0.52±0.01	7.5

***Stichococcus* sp.**	-16.47±0.42	-1.29±0.03	0.98±0.04	7
C. luteoviridis	-22.27±1.87	-1.75±0.15	0.76±0.02	8
C. saccharophila	-14.23±0.78	-1.12±0.06	0.66±0.02	7

Zeta potential values of materials were taken from literature and presented in Table 23. Values presented in the table are approximate values read from zeta potential vs pH diagrams for given material.

Table 23 Zeta potential of materials

Material	Zeta potential [mV]		Reference
	at pH=7	at pH=8	
Polyvinyl chloride (PVC)	-90	-95	Lameiras *et al.*, 2008 [492]
Polyethylene terephthalate glycol modified (PETG)	-85 to -54	n/a	Kirby and Hasselbrink, 2004 [493]
Polyethylene terephthalate (PET)	-100	-115	Lameiras *et al.*, 2008 [492]
Polypropylene (PP)	-80	-90	Lameiras *et al.*, 2008 [492]
Polystyrene (PS) beads	-3	n/a	Piskin *et al.*, 1994 [494]
Quartz crystal microbalance stainless steel grade 316	-90	-110	Hedberg *et al.*, 2013 [495]
polished AISI stainless steel grade 304	-15	-10	Boulange-Petermann *et al.*, 1995 [496]
pickled AISI stainless steel grade 304	-50	-60	Boulange-Petermann *et al.*, 1995 [496]
glass	-120	-125	Lameiras *et al.*, 2008 [492]

D.3 Chapter 4: Biofilm growth at aerial conditions

Table 24 *Stichococcus* sp., *C. luteoviridis*, *H. pluvialis* and *C. saccharophila* biofilms growth at aerial conditions on 6 different materials over 5 days cultivation

		Biofilm growth [days] Number of cells in biofilm [cells·10^4/cm^2]		
Strain	Material	1	3	5
Stichococcus sp.	SS 316 BB	160±13	165±7	179±15
	SS 316 SP	110±17	189±21	268±34
	SS 304 BB	154±15	79±31	104±47
	SS 304 SP	89±33	141±17	211±15
	PETG	181±29	174±21	85±7
	PS	326±37	300±62	92±27
C. luteoviridis	SS 316 BB	328±25	547±43	633±32
	SS 316 SP	203±15	342±32	490±28
	SS 304 BB	294±17	657±42	1103±112
	SS 304 SP	300±7	620±59	880±27
	PETG	210±19	312±16	351±8
	PS	332±134	382±23	460±17
H. pluvialis	SS 316 BB	271±19	556±18	707±40
	SS 316 SP	242±17	527±54	621±19
	SS 304 BB	157±12	175±13	227±59
	SS 304 SP	106±11	139±20	180±37
	PETG	171±14	214±35	305±44
	PS	293±40	319±28	421±15
C. saccharophila	SS 316 BB	285±10	208±5	260±19
	SS 316 SP	381±14	361±42	1075±149
	SS 304 BB	251±35	36±32	82±27
	SS 304 SP	137±12	186±36	289±48
	PETG	244±14	402±15	521±50
	PS	304±12	445±20	594±24

D.4 Chapter 5: Growth and detachment of *Stichococcus* sp. and *C. saccharophila* biofilm

Table 25 Number of cells and detachment energy for *Stichococcus* sp. and *C. saccharophila* biofilms on PETG and PS

	Number of cells [cells·10^4/cm^2]				Detachment energy (kJ/m^2)			
	Stichococcus sp.		*C. saccharophila*		*Stichococcus* sp.		*C. saccharophila*	
Day	PETG	PS	PETG	PS	PETG	PS	PETG	PS
1	148±10	243±11	83±7	125±11	43.8±4.9	282.2±9.7	33.2±3.8	166.7±19.9
4	144±25	232±11	368±28	309±21	31.6±2.4	150.9±43.8	18.4±1.5	47.6±2.8
7	254±14	222±14	386±30	324±22	9.7±0	63.3±14.6	29.5±4.4	78.7±8.4
10	493±31	413±22	570±44	479±32	21.9±2.4	58.6±0.2	20.3±1.6	68.0±5.4
14	602±47	494±23	959±74	805±55	9.5±1.7	48.0±3.7	20.9±1.1	48.3±5.8
21	654±34	572±87	1103±85	863±59	6.8±1.9	37.8±9.3	11.4±2.1	23.8±2.4
28	654±36	575±17	1183±91	894±61	6.1±1.4	19.5±5.2	9.7±0.2	15.7±2.6
35	660±20	286±13	1028±79	676±46	6.4±1.4	13.5±5.6	5.8±1.1	12.5±2.6

D.5 Chapter 5: EPS and SMP extraction by *Stichococcus* sp. and *C. saccharophila*

Table 26 EPS and SMP secretion by *Stichococcus* sp. and *C. saccharophila* biofilms on PETG

EPS and SMP Concentration [mL/cm^2] on PETG						
	Stichococcus sp.				*C. saccharophila*	
Day	SMP glucose	EPS glucose	SMP protein	EPS protein	SMP glucose	EPS glucose
1	0.019±0.008	0	0.010±0.001	0.008±0.000	0.040±0.006	0
4	0.029±0.006	0.049±0.003	0.013±0.000	0.013±0.000	0.079±0.011	0.027±0.006
7	0.055±0.005	0.045±0.003	0.016±0.001	0.014±0.001	0.088±0.005	0.030±0.002
10	0.070±0.003	0.067±0.007	0.017±0.000	0.015±0.000	0.106±0.043	0.066±0.005
14	0.058±0.004	0.061±0.004	0.027±0.001	0.021±0.001	0.170±0.001	0.026±0.004
21	0.052±0.003	0.019±0.01	0.022±0.000	0.019±0.001	0.200±0.002	0.020±0.007
28	0.029±0.003	0	0.014±0.000	0.008±0.001	0.110±0.007	0
35	0.012±0.006	0	0.011±0.000	0.010±0.000	0.074±0.003	0

Table 27 EPS and SMP secretion by *Stichococcus* sp. and *C. saccharophila* biofilms on PS

EPS and SMP Concentration [mL/cm^2] on PS						
	Stichococcus sp.				*C. saccharophila*	
Day	SMP glucose	EPS glucose	SMP protein	EPS protein	SMP glucose	EPS glucose
1	0.030±0.002	0	0.010±0.001	0.006±0.000	0.069±0.008	0
4	0.011±0.001	0.023±0.002	0.015±0.001	0.013±0.000	0.067±0.005	0
7	0.021±0.015	0.045±0.004	0.017±0.000	0.013±0.001	0.095±0.002	0.019±0.010
10	0.040±0.003	0.024±0.003	0.019±0.001	0.013±0.000	0.139±0.004	0.047±0.006
14	0.075±0.031	0.009±0.000	0.022±0.003	0.009±0.001	0.191±0.008	0.016±0.001
21	0.083±0.006	0	0.020±0.001	0.011±0.000	0.153±0.015	0
28	0.039±0.008	0	0.016±0.001	0.012±0.001	0.084±0.006	0
35	0.028±0.006	0	0.017±0.000	0.011±0.001	0.047±0.013	0

D.6 Chapter 6: Work of attachment

Table 28 Work of attachment of *H. pluvialis*, *C. saccharophila*, *Stichococcus* sp. and *C. luteoviridis* on 9 different materials

		Work of attachment [mJ/m^2]			
No	Material	*H. pluvialis*	*C. saccharophila*	*Stichococcus* sp.	*C. luteoviridis*
1	SS 316 BB	51.9	31.9	14.3	58.1
2	SS 316 SP	47.5	27.5	11.2	51.5
3	SS 304 BB	40.3	24.5	10.7	44.8
4	SS 304 SP	52.4	31.2	13.3	57.7
5	glass	27.9	15.7	6.1	29.8
6	PVC	67.9	41.2	18.1	75.5
7	PETG	70.8	41.8	17.5	77.6
8	PP	40.2	24.6	11.0	44.9
9	PS	74.5	44.2	18.7	81.8

D.7 Chapter 6: Monod kinetic model

Table 29 Theoretical biofilm development of *Stichococcus* sp. on PETG and PS, calculated based on nutrient-dependent growth kinetics in relation to experimental growth

Day	BBM [mg/L]		Theoretical N-dependent growth [cells·10^4/cm^2]		Theoretical P-dependent growth [cells·10^4/cm^2]		Experimental growth [cells·10^4/cm^2]	
	TN	TP	PETG	PS	PETG	PS	PETG	PS
1	59.50	35.07	148	243	148	243	148±10	243±11
4	59.34	34.35	206	332	203	327	144±25	232±11
7	59.19	33.63	287	453	278	438	254±14	222±14
10	59.03	32.91	400	617	378	585	493±31	413±22
14	58.82	31.95	623	932	568	856	602±47	494±23
21	58.45	30.26	1346	1915	1134	1632	654±34	572±87
28	58.08	28.58	2900	3923	2214	3048	654±36	575±17
35	57.71	26.90	6236	8020	4224	5573	660±20	286±13

Table 30 Theoretical biofilm development of *C. saccharophila* on PETG and PS, calculated based on nutrient-dependent growth kinetics in relation to experimental growth

Day	BBM [mg/L]		Theoretical N-dependent growth [cells·10^4/cm^2]		Theoretical P-dependent growth [cells·10^4/cm^2]		Experimental growth [cells·10^4/cm^2]	
	TN	TP	PETG	PS	PETG	PS	PETG	PS
1	59.50	35.07	148	243	148	243	148±10	243±11
4	59.34	34.35	206	332	203	327	144±25	232±11
7	59.19	33.63	287	453	278	438	254±14	222±14
10	59.03	32.91	400	617	378	585	493±31	413±22
14	58.82	31.95	623	932	568	856	602±47	494±23
21	58.45	30.26	1346	1915	1134	1632	654±34	572±87
28	58.08	28.58	2900	3923	2214	3048	654±36	575±17
35	57.71	26.90	6236	8020	4224	5573	660±20	286±13

D.8 Chapter 7: Growth in HBBR and reference reactors

Table 31 Biofilm and culture densities in HBBR, WBBR, SBBR and SPBR

Day	Biofilm density [cells·10^4/cm^2]			Culture density [cells·10^4/ml]
Reactor	HBBR	WBBR	SBBR	SPBR
Inoculum	155±18	155±18	155±18	155±18
1	71±11	71±11	71±11	163±11
2	104±8	N/A	N/A	N/A
3	139±11	N/A	N/A	N/A

7	175±16	83±11	78±23	244±14
14	194±13	118±12	182±43	299±92
21	147±22	221±37	251±26	451±94
28	168±11	705±87	316±12	476±16
35	121±12	628±139	238±38	455±50
42	163±23	305±44	244±11	509±51

Table 32 Growth rates in HBBR, WBBR, SBBR and SPBR

Day	Biofilm density [cells·10^4/cm^2]			Culture density [cells·10^4/ml]
Reactor	HBBR	WBBR	SBBR	SPBR
7	0.15	0.02	0.01	0.07
14	0.08	0.04	0.07	0.04
21	0.04	0.06	0.06	0.05
28	0.03	0.08	0.06	0.04
35	0.02	0.06	0.04	0.03
42	0.02	0.04	0.03	0.03