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Towards the novel concept of microalgae production in surfactant-stabilized foams in a liquid foam-bed photobioreactor

Memoria para optar al grado de doctora
presentada por:

María Vázquez Toscano

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María Cuaresma Franco

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Tesis Doctoral - PhD Thesis

AVANCES EN EL NOVEDOSO CONCEPTO DE PRODUCIR MICROALGAS EN ESPUMAS ESTABILIZADAS POR SURFACTANTES EN UN FOTOBIORREACTOR DE ESPUMAS LÍQUIDAS

TOWARDS THE NOVEL CONCEPT OF MICROALGAE PRODUCTION IN SURFACTANT-STABILIZED FOAMS IN A LIQUID FOAM-BED PHOTOBIOREACTOR

Memoria para optar al grado de doctora en Biotecnología presentada por:

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Huelva, 2020

A mi maravillosa madre.

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> ***If you want to go fast, go alone,***
> ***If you want to go far, go together.***
>
> *African proverb*

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Contents

Abbreviations

3D	Three-dimensional
ABC	ATP-binding cassette
AP	Alkylphenol
APE	Alkylphenol Ethoxylates
BSA	Bovine Serum Albumin
CMC	Critical Micelle Concentration
CTAB	Cetyltrimethylammonium bromide
d.f.	Dilution factor
DW	Dry weight
EO	Ethoxylated
EPS	Exopolysaccharides
FAE	Fatty alcohol ethoxylates
FAME	Fatty acid methyl ester
FDA	Food and Drug Administration
FID	Flame ionization detector
F_v/F_m	Maximal photosynthetic efficiency of Photosystem II
HPLC	High performance liquid chromatography
k_La	Volumetric mass transfer coefficient
LB	Luria-Bertani
LED	Light-emitting diodes
LF-PBR	Liquid foam-bed photobioreactor
MTAB	Myristyltrimethylammonium bromide
MUFA	Monounsaturated fatty acid
N-starved	Nitrogen-starved
N-replete	Nitrogen-replete
N/A	Not assessed
N/P	Nitrogen to phosphorous ratio
NF	Nitrogen fertilizer
NPK	Nitrogen-Phosphorous-Potassium fertilizer
O.D.	Optical density
P.I.	Product information sheet
PLA	Polylactic acid
PPO	Poly (propylene oxide)

PEO	Poly (ethylene oxide)
PSII	Photosystem II
PUFA	Polyunsaturated fatty acid
SD	Standard deviation
SDS	Sodium Dodecyl Sulfate
SEM	Scanning electron microscopy
SPD	Spectral Power Distribution
TCA	Trichloroacetic acid
TGA	Tryptone Glucose Agar
UALse	Urea amidolyase
v/v	volume/volume
w/w	weight/weight

Resumen

Las microalgas son una fuente prometedora y renovable de biocombustibles y de productos con aplicación en campos como la cosmética o la alimentación humana y animal. Sin embargo, los aún altos costes de producción limitan el número de aplicaciones de estas a escala industrial. En la actualidad, las microalgas se producen en fotobiorreactores abiertos o cerrados donde las células crecen en suspensión en cultivos líquidos, pero sistemas de cultivo alternativos deberían ser considerados para conseguir la rentabilidad económica de más procesos de producción basados en microalgas. En este sentido, el cultivo en espuma estabilizada por surfactantes podría convertirse en una alternativa adecuada para la producción económica de microalgas y sus productos derivados. Este novedoso concepto de cultivo ofrece ciertas ventajas con respecto a los sistemas convencionales, resultando en última instancia en una reducción del consumo de agua y energía. Sin embargo, por su novedad, aún son múltiples los interrogantes que rodean a su aplicabilidad. En este sentido, esta Tesis trata diversos aspectos de este novedoso concepto de cultivo.

En primer lugar, esta Tesis se centró en la selección de combinaciones de microalgas y surfactantes con potencial para ser empleadas en un fotobiorreactor de espuma (LF-PBR). Para ello, se establecieron grupos de criterios para seleccionar independientemente adecuadas microalgas (**Capítulo 3**) y surfactantes (**Capítulo 4**). Entre dichos criterios se incluyen las propiedades espumantes de microalgas y surfactantes así como la biodegradabilidad y toxicidad de estos últimos. De acuerdo a estos criterios, el cultivo de *Chlorella sorokiniana* en espumas estabilizadas por Pluronic F68 mostró el mayor potencial de un total de 6 microalgas y 10 surfactantes evaluados.

La biodegradabilidad de varios surfactantes por las bacterias asociadas de forma natural a cultivos de microalgas se investigó con más detalle (**Capítulo 5**). Como resultado, se concluyó que los surfactantes biodegradables no son adecuados para su aplicación en el LF-PBR debido a su incapacidad de mantener una producción estable de espuma.

Por otro lado, podría esperarse que el cultivo de microalgas en espuma alterase la calidad de la biomasa y sus futuras aplicaciones debido a posibles interacciones entre los surfactantes y las células y/o debido a las particulares características

impuestas por el cultivo en espuma (p. ej. alta disponibilidad de CO_2). Por ello, se evaluaron los efectos del Pluronic F68 sobre la composición bioquímica de *C. sorokiniana* en cultivos líquidos y en espuma (**Capítulo 7**). Para esto último se diseñó y construyó un LF-PBR a escala de laboratorio, fácil de construir y de operar (**Capítulo 6**). En este LF-PBR *C. sorokiniana* mostró un crecimiento mucho más rápido en cultivos en espuma comparado con cultivos líquidos, así como una distintiva composición bioquímica y una respuesta al estrés incrementada. Nuestros resultados muestran que, más allá del efecto del surfactante, las condiciones físico-químicas impuestas por el cultivo en espuma afectan al metabolismo de *C. sorokiniana*.

Además, se realizó una primera aproximación sobre la idoneidad del uso de medios de cultivo basados en fertilizantes (**Capítulo 8**), lo cual podría reducir aún más los costes de producción. La selección de un fertilizante NPK adecuado parece ser una herramienta prometedora para alcanzar la producción sostenible de microalgas y sus productos derivados en espumas líquidas.

Por último, dado que los surfactantes pueden actuar como extractantes de metabolitos y agentes líticos, también se investigó y demostró la biocompatibilidad del Pluronic F68 y *C. sorokiniana* (**Capítulo 9**).

En conjunto, los resultados obtenidos en esta Tesis apuntan a un alto potencial del LF-PBR para producir biomasa de microalgas con un perfil bioquímico distintivo y de forma económica y, además, trata las posibilidades y retos de este novedoso concepto de cultivo de microalgas.

Abstract

Microalgae are a promising renewable feedstock for biofuels and chemicals with a variety of market applications, including cosmetics, health, human food and animal feed. However, the commercial applications of microalgae on an industrial scale are limited by the high production costs. Microalgae are currently produced in open and closed photobioreactors where the algal cells grow in liquid suspensions, but alternative cultivation systems should be taken into consideration in order to achieve the economic feasibility of additional microalgae-based production processes. In this sense, cultivation in surfactant-stabilized foams could become a suitable alternative for cost-effective production of microalgal biomass and derived products. This novel cultivation concept offers several advantages over the conventional cultivation systems, which ultimately result in reduced water and energy consumption. However, due to its novelty, many questions on its applicability remain unanswered. In this sense, this Thesis provides insight on several aspects of this novel microalgae cultivation concept.

First, this Thesis focused on the selection of microalga-surfactant combinations with potential to be employed in a liquid foam-bed photobioreactor (LF-PBR). For this, sets of criteria to independently select suitable microalgal strains (**Chapter 3**) and surfactants (**Chapter 4**) were established. These criteria included the foaming properties of the microalgal suspensions and the surfactants, the resistance of the surfactant to biodegradation and its toxicity to microalgae. From a total of 6 algal strains and 10 surfactants investigated, cultivation of *Chlorella sorokiniana* in Pluronic F68-stabilized foams showed the largest potential according to all the aforementioned selection criteria. Besides, the biodegradability of several surfactants by bacteria naturally associated to microalgae was investigated in more detail (**Chapter 5**). As a result, it was concluded that biodegradable surfactants are not suitable for their application in a LF-PBR due to their inability to maintain a stable foam production.

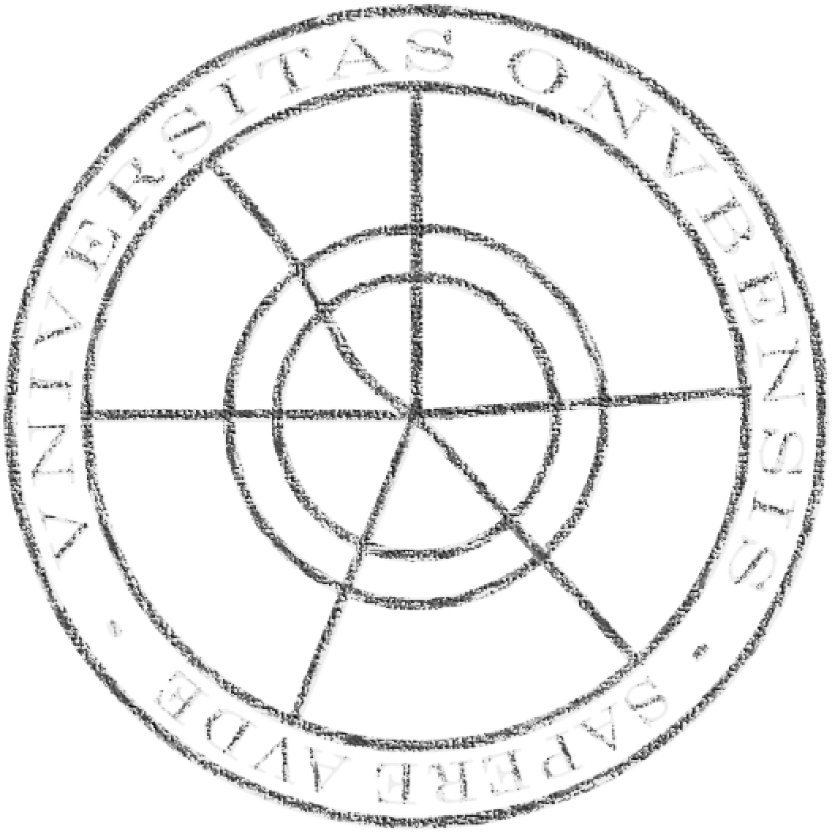
Furthermore, microalgae cultivation in surfactant-stabilized foams was expected to potentially impact the quality of the biomass and its posterior applications due to possible interactions of the surfactant with the cells and/or the particular characteristics imposed by the "foam environment" (i.e. high CO_2 availability). To assess this, the effects of Pluronic F68 on the biochemical composition of *C.*

sorokiniana were investigated in liquid and foam-based cultures (**Chapter 7**). For the later, a simple, easy-to-build and easy-to-operate lab-scale LF-PBR was designed and constructed (**Chapter 6**). Cultivation of *C. sorokiniana* in this LF-PBR showed much more rapid growth compared to liquid cultures, biomass with a distinct biochemical composition and an increased stress response. Our results indicate that, beyond the surfactant effect, the physicochemical conditions of the "foam environment" have an effect on the metabolism of *C. sorokiniana*.

Besides, a first approach was made to assess the feasibility of using a fertilizer-based culture medium (**Chapter 8**), which could reduce the production costs further. The selection of an appropriate NPK fertilizer seems to be a promising tool to achieve sustainable production of microalgae and derived products in liquid foams.

In addition, since surfactants can act as metabolites extractants and lytic agents, the biocompatibility of Pluronic F68 was investigated and demonstrated for *C. sorokiniana* (**Chapter 9**).

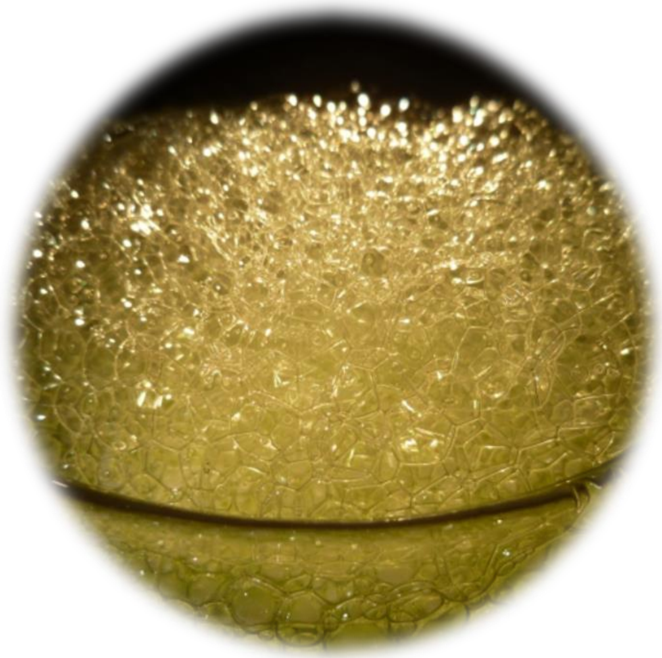
Overall, the results obtained in this Thesis indicate a high potential of the LF-PBR for cost-effective microalgal biomass production with distinct metabolites profile and it gives a first insight on the main potentialities and challenges of this novel algal cultivation concept.



I. INTRODUCTION

Chapter 1

General introduction and Thesis outline



1.1 Microalgae in the circular bioeconomy

The new circular economy frame requires institutions, companies and citizens to change mind and daily act in order to harmonize personal habits and professional processes and procedures with a more sustainable way of acting as a society. As defined by Geissdoerfer et al., (2016), circular economy is an economic system aimed at minimizing waste and making the most of resources. In such system, resource input and waste, emission, and energy leakage are minimized by slowing, closing and narrowing energy and material loops. To do this, long-lasting design, maintenance, repair, reuse, remanufacturing, refurbishing and recycling are key operational aspects (Geissdoerfer et al., 2016). This new model is in contrast to the traditional production model consisting of taking, making and disposing (Ellen MacArthur Foundation, 2012). Besides, due to the increasing demand for fossil fuel-derived products and, in order to reduce our environmental footprint, the current fossil fuel-based economy should shift towards a biobased economy, which relies on the use of renewable biological sources to produce food, materials, and energy without leaving any impact on the environment (Mohan et al., 2016).

In this context, microalgae emerged in the last decade as an outstanding renewable raw material with applications in many fields, whose production and derived products are perfectly in tune with the new circular bioeconomy frame. A large number of applications of microalgal biomass and its derived products has been investigated and their actual potential for implementation at industrial scale has, in some of the cases, been assessed. This is the case for biodiesel from microalgae, whose production process from promising microalgal species was technically developed, although it has so far been considered economically unfeasible (Rawat et al. 2013). Besides, other microalgal products and applications have attracted attention based on their higher economic value. This is the case for valuable products obtained from microalgae for cosmetics and health, human food and animal feed (Forján et al., 2015). Above all, high value molecules with impact in human health are definitely targeted nowadays in microalgal biotechnology for further production processes implementation at industrial scale. Regarding the application as food or feed ingredient, and based on the microalgal nutritional properties which depend on species composition and growth conditions (Guedes and Malcata, 2012; Liang et al., 2018), a range of active compounds have been obtained, such as polyunsaturated fatty acids (PUFAs), carotenoids and other pigments, vitamins, proteins and saccharides (Forján et al., 2015). Such microalgal compounds have been reported to display beneficial activities to human health. For instance, there is a noticeable number of studies that prove those compounds to exert anticancer, anti-inflammatory, antimicrobial and even hypotriglycerydemic and hypocholesterolemic activities, thus increasing these compounds value as potential nutraceuticals (de Vera et al., 2018; Navarro et al., 2016). Microalgae have also increased their value in the feed industry as they improve the immune response of fish (Camacho-Rodríguez et al., 2018). In addition to the abovementioned applications, microalgae can be used as crops biofertilizer or in treatment processes of liquid wastes (Forján et al., 2015).

However, improved production and processing techniques are still required to lower microalgal biomass production costs at industrial scale. Production of valuable molecules from microalgae requires massive production of biomass, which is a mandatory task for any biotechnological application of microalgae at large scale. Currently, large scale microalgae production is performed in open or closed photobioreactors, making use of large water volumes in all cases. Managing large water volumes results in high energy costs (Norsker et al., 2011), which can be even higher by harvesting the produced biomass by industrial centrifugation. It is noteworthy that harvesting costs can be reduced by using other techniques such as flocculation, tangential filtration or foam flotation. Foam flotation has been studied and used to harvest microalgal biomass (Alhattab and Brooks, 2017; Chen et al., 1998; Coward et al., 2013, 2014, 2015; Garg et al., 2015; Shen et al., 2018), but a new application has emerged which combines the use of liquid foams for microalgae cultivation and a possible partial harvesting of the produced biomass (Janoska et al., 2017). Therefore, cultivation of microalgae in foam could present an alternative system to produce algal biomass and derivate valuable products that might solve some of the abovementioned limitations in a combined manner. Within this cost-effectiveness frame, the use of surfactants for microalgal cultivation in liquid foams emerges as a novel promising strategy and several key aspects of the microalgae cultivation in foam concept have been studied in this Thesis.

1.1.1 Microalgae cultivation in liquid foams

The concept of cultivating microalgae in liquid foams appeared as an attempt to reduce costs of microalgae-based production processes (Janoska et al., 2017). This idea is based on the formation of microalgal-enriched liquid foam, which is essentially a complex network of interconnected liquid channels containing the algal cells and surrounding the gas bubbles (**Fig. 1.1**).

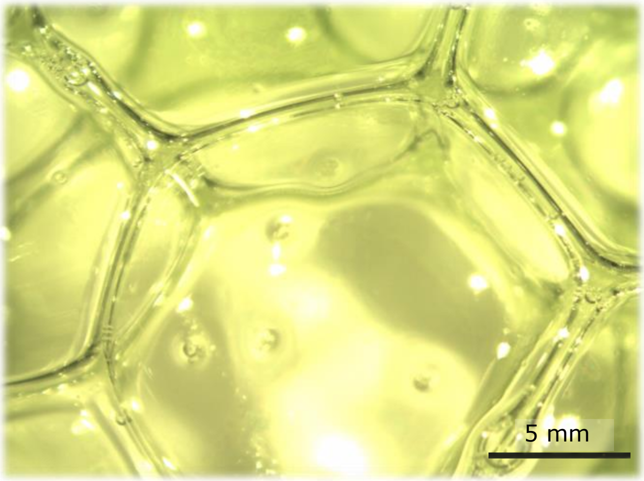


Figure 1.1. *Chlorella sorokiniana*-enriched foam observed under a magnifying glass at a magnification of 300.

Algal cultivation in foam presents several advantages compared to the conventional liquid-based cultivation. First, the increased interfacial area results in enhanced transfer of CO_2/O_2 between the gas (initially enriched in CO_2) and the liquid phases (containing the algal cells). This has been already demonstrated by Janoska and co-workers (2018a), who reported 6-fold higher volumetric mass transfer coefficients (k_La) in a photobioreactor operated as a LF-PBR compared to the same system operated as a bubble column. Regarding CO_2 transfer, k_La is the most commonly used parameter to assess the performance of microalgae cultivation systems (Ugwu et al., 2008). Besides, the long residence time of the gas bubbles led to high CO_2 uptake efficiencies. So far, the only value reported in a LF-PBR goes up to 97% for the CO_2 uptake (Janoska et al., 2018b), which is higher than most reported values for other microalgae cultivation systems such as open ponds (Putt et al., 2011; Tsai et al., 2017), tubular PBRs (Sobczuk et al., 2000), open-thin layer systems (Doucha et al., 2005) or biofilm-based cultivation (Blanken et al., 2017). Next, the short light absorption path, combined to the abovementioned increased mass transfer and the proper nutrient supply, allows for cultivation at high biomass densities, which results in high biomass productivities and a potential reduction in the harvesting costs. In this sense it has been reported that a LF-PBR can be operated as chemostat at biomass concentrations of 20-25 g L^{-1} (Janoska et al., 2018a). But these values could be enlarged up to 30-60 g L^{-1} according to model predictions (Janoska et al., 2018b), which are much higher than the biomass concentrations usually employed in conventional liquid-based photobioreactors such as open ponds (Borowitzka, 2005) and tubular photobioreactors (Pawar, 2016). The fact that a LF-PBR operates at larger biomass densities could result in avoiding the first step of biomass pre-concentration, commonly referred to as primary or bulk harvesting. This is considered a major advantage since harvesting and dewatering of the microalgal biomass can involve up to 30% of the total process cost (Zhang and Zhang, 2019). In addition, the low liquid fraction of the foam results in lower pressure drops compared to liquid-filled reactors, which could reduce the construction costs of systems at large scale. Due to all the aforementioned advantages, cultivating microalgae in liquid foams offers large energy savings compared to liquid-based cultivation systems. In this sense, the total energy requirement for microalgae production in a LF-PBR and the posterior biomass harvesting has been calculated to be only 8.5% of that in a liquid flat panel photobioreactor (Janoska et al., 2018b).

Altogether, microalgae cultivation in a LF-PBR arises as a promising cultivation system for economically feasible microalgae-based production processes. However, due to the novelty of this concept, there is very little research performed on the fundamental aspects of microalgae cultivation in liquid foams.

1.1.2 Stable and viable microalgal-enriched foam as cultivation matrix

Cultivation of microalgae in a LF-PBR is based on the production of liquid foams which must be highly enriched in microalgae. A general overview of a liquid foam-bed photobioreactor (LF-PBR) is shown in **Figure 1.2**. In order to produce the foam, a small volume of culture suspension containing a surfactant is bubbled with CO_2-enriched air and, using a suitable alga-surfactant combination, algal-enriched foam is formed. The extent at which the foam is enriched with microalgae will depend on the characteristics of the microalgal strain and those of the surfactant employed, but also on the interactions that take place between the surfactant and the algal cells.

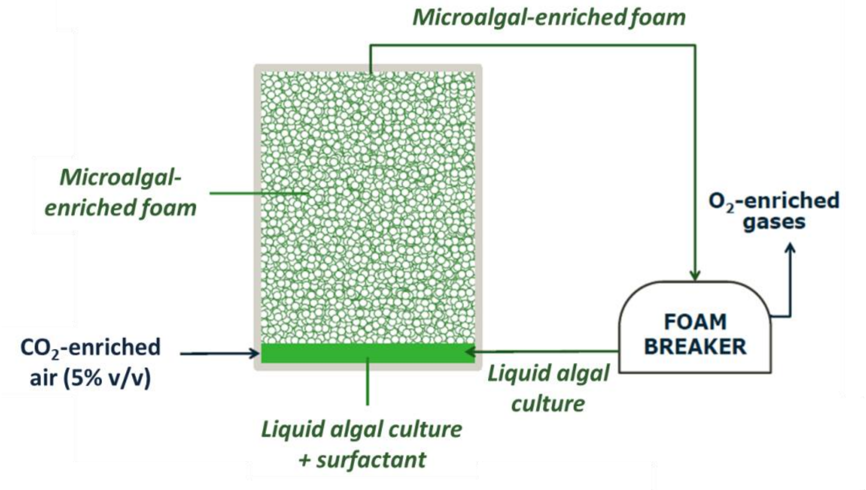


Figure 1.2. General overview of a liquid foam-bed photobioreactor (LF-PBR).

During the foam formation process, the hydrophobic parts of the surfactant molecules stick to the air bubbles, while other interactions with the algal cells have to occur for them to be dragged up (**Fig. 1.3**). For a microalgal cell to be dragged up (i.e. to migrate from the liquid to the foam phase), it has to collide with the bubble and attach to it. In principle, all microalgal strains could be cultivated in a LF-PBR. However, certain traits of the specific algal strains, such as its cell size or the presence of aggregation forms, may affect their probability to be dragged up. Besides, the characteristics of the algal cell surface can have an impact on the suitability of a particular strain to be cultivated in a LF-PBR. In this sense, microalgal cell hydrophobicity has been considered crucial for successful algal-enriched foam production (Garg et al., 2012), and the culture age can in turn modify this characteristic of the cell cover of a particular algal strain (Zhang et al., 2012). Therefore, some particular microalgal cell features have to be taken into consideration in order to select algal strains with potential to be cultivated in a LF-PBR.

In the most favorable scenario, the surfactant needed to produce and stabilize the foam would be produced by the microalgal cells themselves. It is known that some microalgae are source of natural surfactants (Žutić et al., 1981). However, the knowledge on the production of natural surfactants from microalgae is still limited (Rahman et al., 2018) and only a few studies have reported the use of naturally produced surfactants to harvest microalgal biomass via foam flotation (Csordas and Wang, 2004; Levin et al., 1962). For instance, algal exopolysaccharides (EPS) have potential as biosurfactants (Rahman et al., 2018), but these compounds are often secondary products and may therefore not be sufficiently produced and excreted during the active phase of growth. Moreover, for the usage of algal biosurfactants conditions not optimal for growth, such as low pH (Levin et al., 1962) or even disaggregation of the cell wall (Law et al., 2018), may be needed. Therefore, the use of algal biosurfactants would be limited by the capacity of microalgae to produce them, the amount produced and excreted, and the cultivation conditions needed for their production and/or release.

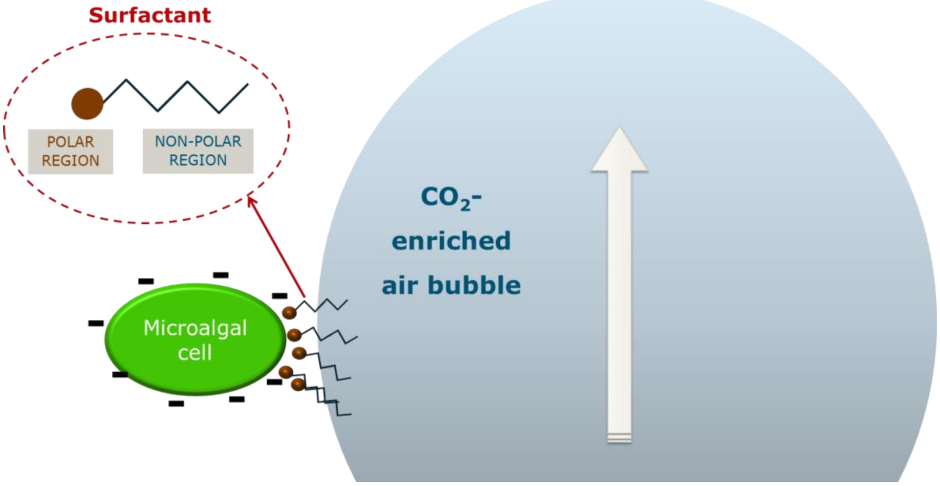


Figure 1.3. Schematic view of a surfactant molecule and the interactions among a microalgal cell, the surfactant molecules and a gas bubble. The red arrow indicates a schematic description of a surfactant structure, zoomed from the main drawing. The minus symbols around the microalgal cell represent the negative charge of the algal cell surface. The white arrow represents the bubble-surfactants-microalgae complex travelling upwards along the foam column.

Considering that the biosurfactants naturally produced by the alga may not be sufficient to sustain foam-based cultivation in a LF-PBR, supplementation of the algal culture with external surfactants appears as a more feasible alternative, since it would make the foam production independent from the excretion of biosurfactants by the own microalga cultivated. So far, surfactants have been investigated to produce algal-enriched foam with the main aim of collecting the biomass via foam flotation (Coward et al., 2013; Liu et al., 1999). For this purpose, as well as for cultivation in a LF-PBR, the surfactant must have good foaming properties and also be a good match for the microalga in terms of microalgae enrichment in the foam. For the latter, the interactions between the microalgal cells and the surfactant molecules are crucial, since surfactants can make microalgal surface more hydrophobic, thus favoring the attachment of the cells to the bubbles and determining the flotation performance (Alhattab and Brooks, 2017; Hao et al., 2017; Liu et al., 1999). However, beyond the needs of a foam flotation process, foam-based cultivation of microalgae requires the continuity of the foam production process in order to allow long-term algal cultivation and also the maintenance of the algal cells viability to allow algal growth in the LF-PBR. Accordingly, the specific chemical characteristics of a surfactant potentially suitable for cultivation in foam should in principle match the specific requirements of the microalgal strain object of cultivation.

The biodegradability of the surfactant is another key aspect regarding the stability of the foam-based cultivation process. The biodegradable character of a surfactant varies largely among surfactant groups and it is directly related to the chemical composition and structure (Ivanković and Hrenović, 2010; Jurado et al., 2007). For instance, surfactants with aromatic rings or highly branched carbon chains possess, in general, lower biodegradation rates (Dorn et al., 1993; Nyberg et al., 1988). Whereas the use of a biodegradable surfactant would carry fewer implications for the final applications of the algal biomass produced, it would also shorten its availability in the LF-PBR, which would result in a continuous need of surfactant addition and could also result in non-desired microorganisms thriving. Accordingly, surfactants with low biodegradation rates would, in principle, be more suitable for stable microalgae cultivation in surfactant-stabilized foam.

On top of all the requirements stated above, the surfactant employed must lack toxic effects on the particular microalga in order to allow its stable cultivation in the LF-PBR. Surfactants can result in a variety of toxic effects on microalgae, including reduction in growth rate (Lechuga et al., 2016), reduction of photosynthetic activity (Azizullah et al., 2014), or can even lead to cell disruption and cell lysis (Lewis, 1990). However, the toxic effects are highly specific of the particular surfactant and the organism tested (Ivanković and Hrenović, 2010), which results in the need of assessing the toxicity of the surfactants and the tolerance of microalgae for each microalga-surfactant combination independently.

For this reason, microalga-surfactant combinations that allow microalgae cultivation in surfactant-stabilized foams without compromising viability are a requirement to perform microalgae cultivation in a LF-PBR.

Thus, in order to find a suitable microalga-surfactant combination that allows stable production of viable microalgal-enriched foam, not only the particular traits of the microalgal strain and the surfactant explained above have to be considered, but also the specific interactions that take place between both of them must be particularly assessed for each potential microalga-surfactant combination.

1.1.3 Microalga-surfactant interactions in a LF-PBR

In a LF-PBR, the algal-enriched foam is stabilized by surfactants, which are molecules with hydrophobic and hydrophilic domains. As explained before, the algal cells grow in the thin liquid layers that surround the gas bubbles and contain the surfactant molecules. A simple scheme of the complex formed by the microalgal cells, the surfactant molecules and the gas bubbles in surfactant-stabilized microalgal-enriched foam, and some interactions expected to take place, are shown in **Figure 1.4**. As microalgal cell surface is in general negatively charged (Phoochinda and White, 2003), algal cells are expected to interact with the hydrophilic domains of the surfactant molecules. On the other hand, bubbles in flotation are considered electronegative and hydrophobic (Kwak and Kim, 2015), therefore favoring their attachment to the surfactant molecules by their hydrophobic ends. Thus, surfactants can act as a bridge between the algal cells and the gas bubbles. It is noteworthy that surfactants can form multilayers between the algal cells and the gas bubbles (Shen et al., 2018), therefore the potential interactions between the algal cells and the surfactant molecules may not be limited to the hydrophilic parts of the latter.

Despite there is limited knowledge on the precise mechanisms behind the interaction between surfactants and algal cells, it is known that the interactions between the surfactants molecules and the microalgal cell covers vary among surfactants. As example, Shen and co-workers (2018) reported the adsorption of two different surfactants, hexadecyltrimethyl ammonium bromide (CTAB) and tea Saponin, on *Chlorella vulgaris* cells. Whereas CTAB bonds the algal cell via neutralization reaction (including hydrogen bonds), hydrophobic attraction and Van der Waals forces are behind the attachment of Saponin onto *Chlorella* cells (Shen et al., 2018).

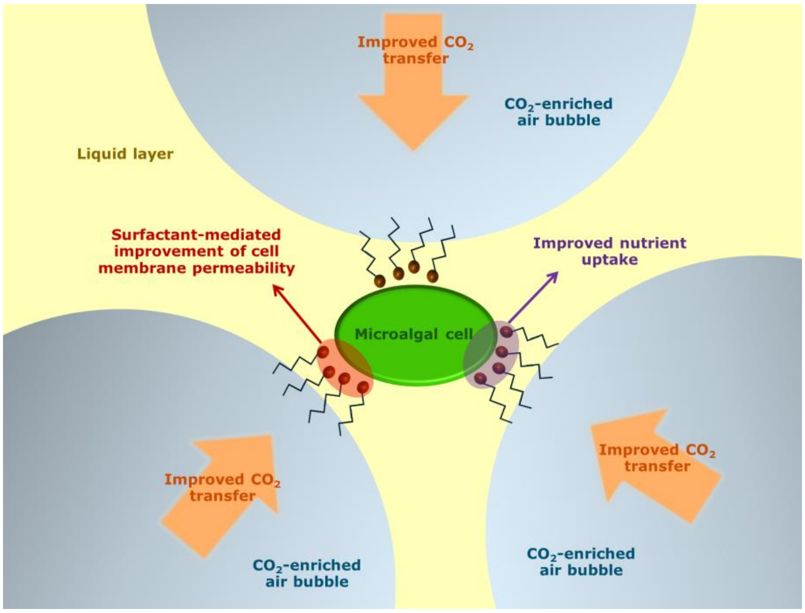


Figure 1.4. Scheme of physicochemical features that might contribute to enhanced growth and/or shifted biochemical composition of microalgae growing in liquid foams. The interactions between the hydrophilic part of the surfactant molecules and a microalgal cell are indicated by the red and purple areas. Possible consequences of these interactions are indicated by the red and purple arrows. The improved CO_2 transfer from the gas bubbles to the algal cell is represented by the orange arrows.

Moreover, the potential interactions between the surfactant molecules and the algal cells might induce changes in the algal cell metabolism, resulting in modified biochemical composition. For instance, the uptake of essential nutrients may be altered by interactions with surfactants (Singh et al., 2016; Taoka et al., 2011), thus leading to differences in growth and/or in the composition of the biomass. As example, a recent study (Esakkimuthu et al., 2019) showed enhanced growth and higher lipid content in the biomass of *Tetradesmus obliquus* when the cultures were supplemented with polysorbitol surfactants as a result of increased membrane permeability and the usage of these surfactants as carbon source.

According to the stated above, long-term interaction of microalgal cells with surfactant molecules during the cultivation in surfactant-stabilized foam might result in changes in the growth pattern and the major biochemical composition of the algal cells and, as a consequence, in biomass of a different quality. In addition, the particular features of the "foam environment", such as the improved CO_2/O_2 transfer, may also play a role in the resulting composition of the produced algal biomass. Consequently, changes in the major biochemical composition of the biomass are expected to occur due to (i) the physicochemical environment of the foam, and (ii) as a result of the continuous interaction of the surfactant molecules and the algal cells.

In order to investigate cultivation in foam, a LF-PBR that allows mid-term cultivation and sufficient cultivation volumes is required. Next to using a suitable microalga-surfactant combination, the successful performance of microalgae cultivation in liquid foams will depend on the LF-PBR configuration and the operational conditions employed. In general, a LF-PBR is composed of (i) a foam production system based on sparging with CO_2-enriched air bubbles, (ii) a vessel containing the foam, where microalgal cells grow in the liquid channels surrounding the gas bubbles, and (iii) a foam-breaker part, where the liquid and gas phases are separated to allow oxygen release (**Fig. 1.2**). In addition, the liquid released from the broken foam, which should be more concentrated in microalgal cells, has to be returned to the initial point where the foam is continuously produced.

So far, two different LF-PBR designs have been developed. The first LF-PBR reported consisted of a flat panel, where foam-bed cultivation occurs, and a packed bed column filled with hydrophobic beds to break the foam (Janoska et al., 2017). Then, a second LF-PBR was designed which showed improved algal enrichment of the foam by the inclusion in the design of a liquid recirculation circuit from the bottom to the top part of the cultivation vessel (Janoska et al., 2018a). The existent LF-PBR designs allowed to prove the concept, to improve the foam formation and foam breaking systems, and they also allowed algal cultivation in surfactant-stabilized foams for longer periods of time. In order to do so, operation at lab-scale with small cultivation volumes were sufficient (i.e. 150 and 450 mL). However, new LF-PBR designs that allow larger cultivation volumes would permit to study this novel cultivation concept at a larger scale. Cheap and easy-to-build LF-PBR systems would also allow to assess different cultivation scenarios and their impact on foam-based cultivated microalgal biomass. For instance, and considering the particular scenario of microalgae cultivation in surfactant-stabilized foams, the impact of applying stress conditions on microalgae may differ when the biomass is foam-based cultivated compared to that in liquid cultures.

1.1.4 Further strategies in foam-based microalgae cultivation

Although microalgae cultivation in liquid foams has been proved to be a promising low-cost alternative to conventional cultivation systems due to the high energy savings in the cultivation and harvesting process (Janoska et al., 2018b), additional improvements could be done in order to reduce production costs further. Regarding the biomass production, sunlight should be efficiently used as energy source in phototrophic cultivation (Cuaresma et al., 2009) and nutrients should be supplied by alternative sources that are cheaper than pure chemicals.

Carbon is the major element in the algal biomass and, in this sense, it has been shown that industrial flue gasses can supply CO_2 for microalgae production (Doucha et al., 2005). Besides carbon, nitrogen, phosphorus, sulfur and a number of other chemical elements including potassium and micronutrients of crucial importance to the algal growth might be supplied in the form of commercially cheap available formula. For instance, the so-called NPK fertilizers, which are composed of a nitrogen source, a phosphorus-containing compound and a potassium source, can substitute the expensive and complex standard culture medium prepared in the laboratory. There is a wide variety of agricultural fertilizers with different ratio between the main elements (i.e. nitrogen, phosphorous and potassium) which can be evaluated to optimize the growth of a particular microalgal species. Similarly, there are commercially available products containing the micronutrients needed for algal growth, which in combination with an adequate NPK fertilizer may provide a suitable and cheaper alternative culture medium for economically feasible and systematic foam-based algae production.

Furthermore, other possibilities of cultivating microalgae in surfactant-stabilized foams have not been explored yet due to the novelty of the concept. For instance, strategies related to the downstream processes posterior to the algal biomass harvesting, this is, cell disruption and the extraction of valuable compounds, when pursued. In this regard, it is known that surfactants can be also employed in cell disruption and as extractants of certain compounds from microalgal cells (Glembin et al., 2014; Park et al., 2014; Ulloa et al., 2012). Thus, the possibility exists that, along the foam-based cultivation, the interactions between the surfactants and the algal cells result in simultaneous extraction of high-value molecules. The surfactant-mediated extraction of valuable compounds during foam-based cultivation may be a drawback if the algal biomass is the desired product. However, this concept of simultaneous cultivation and extraction of valuable compounds, commonly referred as "milking", has been proposed as a cheaper alternative to harvesting and dewatering the biomass and its posterior cell disruption and products extraction. As examples, milking algae has been explored to extract astaxanthin from *Haematococcus pluvialis* (Samori et al., 2019), β-carotene from *Dunaliella salina* (Hejazi et al., 2002; Hejazi and Wijffels, 2004) and hydrocarbons from *Botryococcus braunii* (Frenz et al., 1989) using solvents. In the case of foam-based microalgae cultivation, the valuable compounds would be extracted by the surfactant used to stabilize the foam. Thus, the cultivation conditions (i.e. the particular microalga-surfactant combination used and the surfactant concentration employed) must ensure that no toxic effects are occurring while allowing the extraction of the targeted compounds. So far, it has been reported that simultaneous harvesting and enhancement of the posterior extraction of lipids can be achieved using the surfactant CTAB in foam flotation (Coward et al., 2014), but the possibility of surfactant-mediated extraction of valuable products from microalgae growing in surfactant-stabilized foams remains unexplored.

The feasibility of using commercial fertilizers as culture medium and the surfactant potential to extract high-value compounds are potential strategies to consider in the achievement of sustainable and economically feasible microalgae-based processes in foam.

1.2 Thesis hypotheses and aims

First, according to published research previously commented in the Introduction section, there is scientific knowledge describing main structural and functional features of microalgae cell surfaces and structures and main physicochemical properties of surfactants. Based on that existing knowledge, it was hypothesized that an adequate analysis of those microalgae and surfactants traits should allow to establish criteria that could be broadly applied to select microalga-surfactant combinations suitable for stable production of viable microalgal-enriched foam.

Secondly, considering the particular conditions faced by the algal cells growing in a liquid foam-bed photobioreactor, including the interactions between the surfactant molecules and the algal cells and the physicochemical conditions of the foam, it was hypothesized that such a distinctive scenario should have an effect on the algal growth and on the algal biomass composition.

Finally, according to the still scarce existing knowledge on microalgal cultivation in liquid foams, it was hypothesized that this novel cultivation concept would be expected to be combinable with other strategies to reduce production costs, specially the usage of commercial fertilizers as algal culture medium and the surfactant-mediated extraction of high-value metabolites.

According to the abovementioned hypotheses, this Thesis was aimed at achieving stable production of viable microalgal-enriched foam for the production of microalgae in liquid foam-bed photobioreactors, giving special attention to the microalga-surfactant interactions and their resulting effects on the algal biomass quality. In addition, the combination of microalgae cultivation in liquid foams with the usage of commercial fertilizers and with surfactant-assisted extraction of valuable products were investigated as possible further strategies to achieve the sustainability of microalgae-based production processes in liquid foam-bed photobioreactors.

That main objective would be achieved through the following specific objectives:

1. **To select a microalga-surfactant combination which allows stable production of viable algal-enriched foam**
    1.1. To select suitable microalgal strains for the production of algal-enriched foam
    1.2. To select suitable surfactants for the production of algal-enriched foam
    1.3. To determine the effects of surfactant biodegradability on bacterial and algal populations and on surfactant foaming capacity
2. **To unravel the changes produced in the microalgal biochemical composition derived from the interaction with the surfactant and the distinct physicochemical environment of the liquid foam**
    2.1. To design and construct a simple lab-scale liquid foam-bed photobioreactor to perform experiments in surfactant-stabilized algal-enriched foam
    2.2. To unveil the effects of the surfactant presence and the physicochemical conditions imposed by the cultivation in liquid foams on the growth and biochemical composition of the algal biomass
3. **To explore the combination of foam-based microalgae cultivation with other strategies towards the sustainability of microalgae-based productions processes**
    3.1. To determine the compatibility between the surfactant and the use of commercial fertilizers as culture medium
    3.2. To analyze the potential of the surfactant as downstream agent (i.e. as fatty acids extractant and lytic agent)

1.3 Outline of this Thesis

Several aspects of microalgae cultivation in liquid foams were studied in this Thesis. The selection of a suitable microalga-surfactant combination for its application in a LF-PBR was conducted in **Chapter 3** and **Chapter 4.** In **Chapter 3** a group of criteria to assess the potential of a certain microalgal strain to be cultivated in liquid foams is presented. Using a model surfactant, the assessment of 6 microalgal strains according to the established criteria allowed the selection of the most promising species for further investigation. Besides, the production of foam by the own microalgal cultures was proved insufficient in all cases and the addition of a surfactant was deemed necessary for the production of stable microalgal-enriched foam.

Similarly, certain criteria were established in **Chapter 4** for the selection of a surfactant with potential to be employed in a LF-PBR. Using the microalgal strains selected in Chapter 3, the potential of 10 surfactants according to the proposed criteria was evaluated. As a result, the surfactant with the highest potential was chosen for further studies.

In **Chapter 5** the consequences of using biodegradable surfactants in microalgal cultures were elucidated. The 3 surfactants with the highest biodegradation rates from Chapter 4 were added to microalgal liquid cultures and their effects on both microalgal and bacterial populations are presented. Besides, how the surfactant biodegradation impacts the foaming capacity of surfactant-added microalgal suspensions is shown for a model biodegradable surfactant.

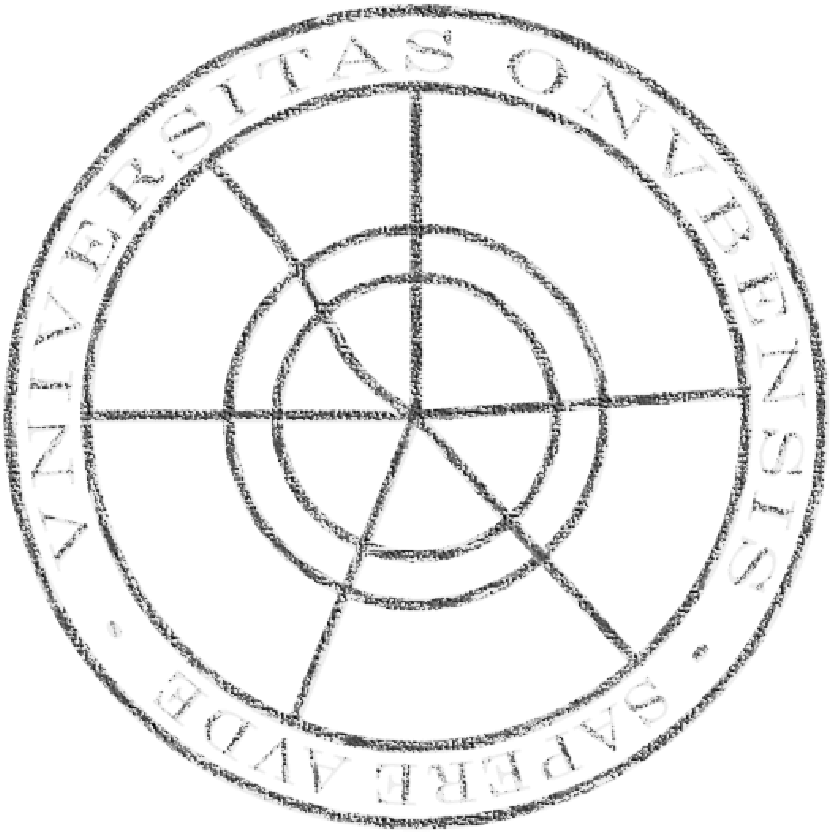
In **Chapter 6** a simple LF-PBR designed to perform lab-scale experiments on microalgal-enriched foam is presented. Mid-term operation of the presented system was achieved using solutions of the previously selected surfactant. The effect of temperature increase due to illumination on the foaming capacity of the surfactant is also described.

In **Chapter 7** the effects of the selected surfactant on microalgal growth and the biochemical composition of the algal biomass in liquid and foam-based cultures, under no stress conditions and subjected to stress conditions (including nitrogen starvation and increased irradiances) were assessed. The potential of LF-PBR for cost-effective microalgal biomass production with distinct metabolites profile is shown.

In **Chapter 8** the adequacy of fertilizer-based culture media in combination with the selected surfactant in terms of microalgal growth and intracellular content of photosynthetic pigments, especially lutein, was evaluated. To do so, several nitrogen fertilizers were assessed as nitrogen source and two potential NPK-based culture media were evaluated. The feasibility of fertilizer-based culture media for cost-effective foam-based microalgae production is shown.

In **Chapter 9** the capability of the selected surfactant to extract fatty acids along foam-based microalgae cultivation under no stress and under different stress conditions is shown. Besides, the effect of the surfactant at high concentrations on the cell integrity and fatty acids extraction is presented.

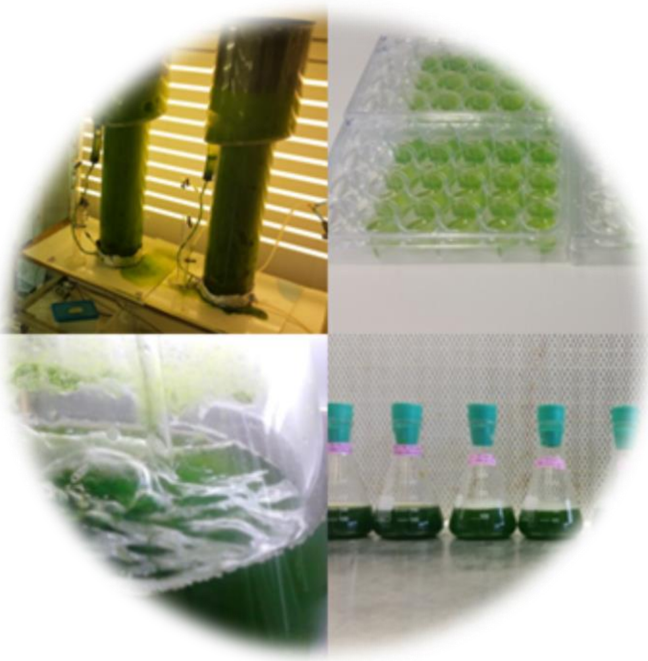
Chapter 10 is a general discussion of the main findings presented in this Thesis and the potential of microalgae production in surfactant-stabilized foams. Future research prospects and challenges still unaddressed are also discussed.



II. MATERIALS AND METHODS

Chapter 2

Materials and Methods



2.1 Microorganisms

2.1.1 Microalgal strains

Scenedesmus obliquus (*S. obliquus*) and *Nannochloropsis gaditana* (*N. gaditana*) were kindly provided by Fitoplancton Marino S.L. (Cádiz, Spain), *Botryococcus braunii* var. Showa (*B. braunii* var. Showa) was obtained from University of California, Berkeley Herbarium; *Botryococcus braunii* CCALA-778 (*B. braunii* CCALA-778) was obtained from Culture Collection of Autotrophic Organisms, Trebon, Czech Republic; and *Chlorella sorokiniana* CCAP 211/8K (*C. sorokiniana*) and *Neochloris oleoabundans* UTEX 1185 (*N. oleoabundans*) were obtained from UTEX culture collection.

2.1.2 *Scenedesmus obliquus*-associated bacteria

The bacteria present in *Scenedesmus obliquus* cultures were isolated by streaking aliquots of the microalgal culture broth on Trypton Glucose Agar (TGA) plates (Ref. 4021452, Biolife, Italia) and incubated at 37 ºC.

Bacterial liquid cultures were prepared in 1-L flasks in Luria-Bertani (LB) medium. The cultures were inoculated with a mix of the morphologically different colonies previously grown in the TGA plates. The flasks were placed in an orbital shaker incubator at 37 ºC and 115 rpm (MaxQ 4000, Thermo Scientific, United States). The cultures were maintained in these conditions for a maximum of a week.

2.2 Chemicals

2.2.1 Surfactants

Ten different surfactants were evaluated for their suitability for the liquid foam-bed photobioreactor. The selected surfactants contain two natural surfactants and 8 synthetic surfactants: six non-ionic (Pluronics F68 and P84, Tergitols NP9 and TMN6, Triton X-100 and Tween 20), one cationic (CTAB), one anionic (SDS). The natural surfactants were Saponin and a reference protein surfactant, Bovine Serum Albumin (Janoska et al., 2017). All the surfactants used are listed in **Table 2.1** and their chemical structure is shown in **Figure 2.1**.

Table 2.1. Summary of the surfactants used in this Thesis. P.I. stands for the online product information sheets.

Surfactant	Source details	Type	CMC (g L^{-1})	Ref. CMC	Chemical composition
BSA	Sigma A7030	Non-catalytic protein, anionic character	0.03	Clarkson et al., 1999b	Bovine Serum Albumin derived from cows
CTAB	Sigma H5882	Cationic	0.346	Sigma P.I., middle of given range	Cetyltrimethylammonium bromide
Pluronic F68	Panreac A1288	Non-ionic	0.334	Sigma Aldrich P.I.	Triblock copolymers of ethylene and propylene oxide
Pluronic P84	Sigma 713538	Non-ionic	0.298	Kozlov et al., 2000	Triblock copolymers of ethylene and propylene oxide
Saponin	Sigma 84510	Non-ionic	0.05	Sigma P.I., middle of given range	Glycoside+ Triterpene
SDS	Sigma L3771	Anionic	2.451	Sigma P.I., middle of given range	Sodium dodecyl sulfate
Tergitol NP9	Sigma 521078	Non-ionic	0.06	DOW P.I.	Nonylphenolethoxylate
Tergitol TMN6	Sigma 60828	Non-ionic	0.8	DOW P.I.	Branched secondary alcohol ethoxylate
Triton X-100	Panreac A1388	Non-ionic	0.194	Applichem P.I.	Octyl phenol ethoxylate
Tween 20	Sigma, P1379	Non-ionic	0.06	Helenius et al., 1979	Polyoxyethylene (20) sorbitanmonolaurate

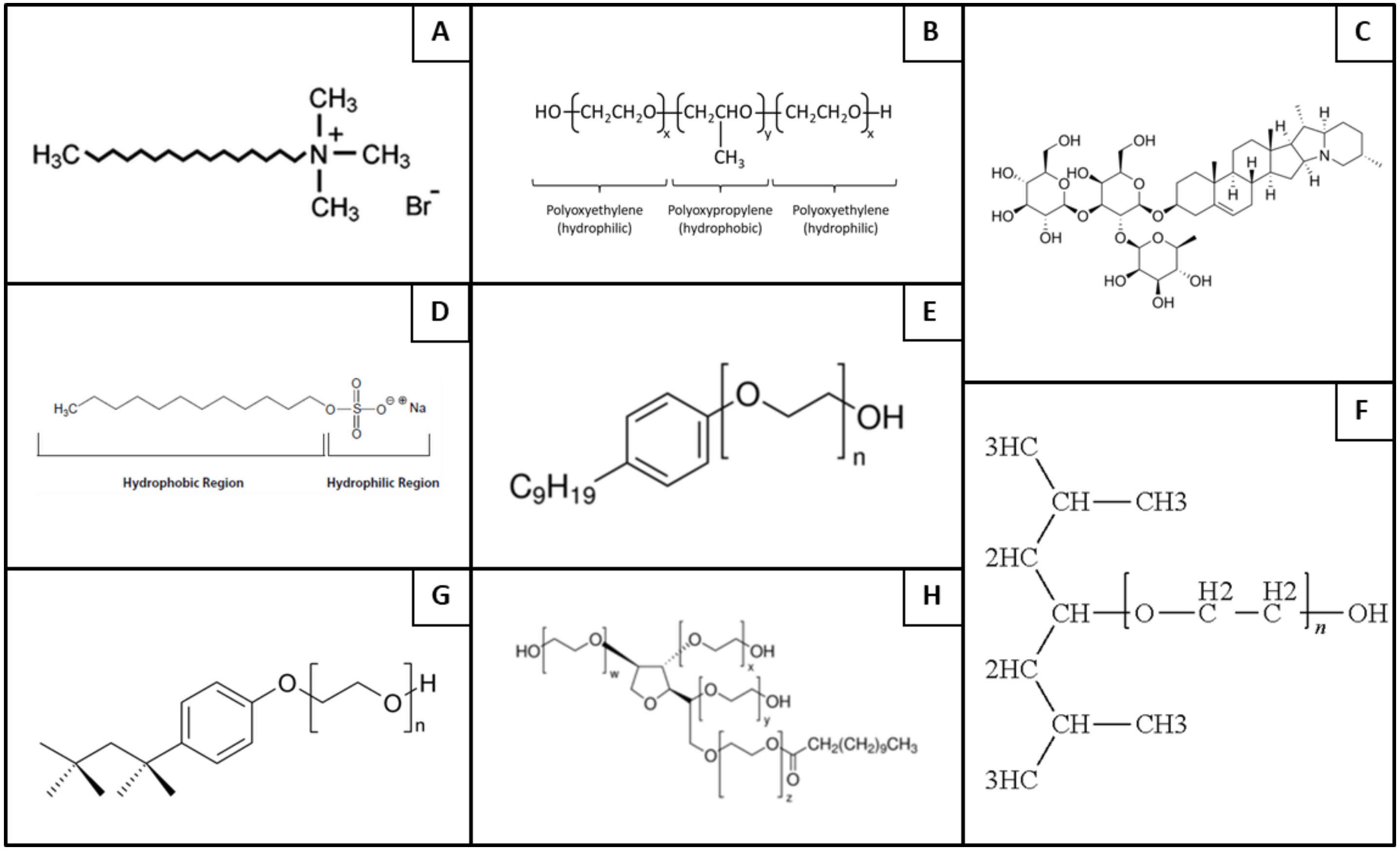


Figure 2.1. Chemical structures of surfactants. (A) Hexadecyltrimethylammonium bromide (CTAB), (B) Pluronics, (C) Saponin, (D) SDS, (E) Tergitol NP9, (F) Tergitol TMN6 (n~8), (G) Triton X100, (F) Tween 20.

The comparison of these surfactants was done at specific surfactant concentrations, taking into account their critical micelle concentration (CMC). The CMC is the concentration above which the surfactant molecules start forming micelles in the liquid phase. The CMC values of the different surfactants are included in **Table 2.1**. These CMC values were collected from literature and/or the product information sheets (P.I.), where they are reported or assumed to have been determined at room temperature.

2.2.2 Nitrogen fertilizers

In Chapter 8, 10 different nitrogen fertilizers were used as a replacement of the original nitrogen source of the M-8 culture medium. They were added to nitrogen-free M-8 culture medium at the corresponding concentration in order to approximate the original nitrogen concentration of the medium, 0.03 M. Their compositions are shown in **Table 2.2**.

Table 2.2. Nitrogen fertilizers (NFs) and their composition in terms of the form in which nitrogen is provided.

		Nitrogen form (% w/w)		
Fertilizer code	**Chemical composition**	**Nitrate**	**Ammonium**	**Urea**
NF1	Potassium nitrate	100	-	-
NF2	Magnesium nitrate	100	-	-
NF3	Ammonium sulfate	-	100	-
NF4	Ammonium thiosulfate	-	100	-
NF5	Ammonium polyphosphate	-	100	-
NF6	Chemicals mix	50	50	-
NF7	Chemicals mix	25	25	50
NF8	Chemicals mix	11.15	20.77	68.08
NF9	Chemicals mix	22.86	31.43	45.72
NF10	Urea solution	-	-	100

2.2.3 NPK fertilizers

Several agricultural NPK fertilizers were used in Chapter 8 in order to assess the adequacy of NPK-based culture media for *C. sorokiniana* cultivation. A NPK fertilizer is an agricultural fertilizer that contains, at least, nitrogen, phosphorus and potassium, although other elements and nutrients, such as sulfur, can be included in their composition. In general, a NPK is named using a numerical code that indicates its composition, as follows: **NPK a**-**b**-**c** (**d**), where **a, b**, **c**, and **d** refers to the content of N, P_2O_5, K_2O and SO_3, respectively, expressed in % (w/w).

For each case, the NPK concentration is indicated along the text. The NPK fertilizers employed in this Thesis (Chapter 8) were referred to by their numerical code **NPK a-b-c**. Their corresponding tradenames were: Agroliq NPK 18-6-6 (NPK 18-6-6), Agroliq NPK 12-6-4 (3) (NPK 13-6-4) and Agroliq NPK 8-6-6 (NPK 8-6-6). All of them were produced by Agralia Fertilizantes S.L., (Spain) and contained urea as the main nitrogen source, complemented with ammoniacal nitrogen in a lower proportion. The ureic and ammoniacal nitrogen concentrations in the different NPK fertilizers were, respectively: 15.9 and 2.1% (w/w) for NPK 18-6-6, 9 and 3% (w/w) in NPK 12-6-4 and 6 and 3% (w/w) in NPK 8-6-6.

When indicated, an agricultural micronutrient solution (AGRALIA AG-Complex, Agralia Fertilizantes S.L., Spain) was added to the NPK-based culture media. This solution contains: 4.00% (w/w) of Fe, 2.00% (w/w) of Mn, 0.50% (w/w) of Zn, 0.12% (w/w) of Cu, 0.30% (w/w) of Bo and 0.08% (w/w) of Mo. When indicated, NPK-based culture media were also supplemented with 0.4 g L^{-1} of $MgSO_4$ in order to equal the sulfur concentration of the media to that of the reference medium (M-8).

2.3 Experimental conditions

2.3.1 Cultivation conditions for the microalgal strains maintenance

Unless otherwise indicated, the culture media employed were: (i) modified BG11 (Bosma et al., 2008) for *S. obliquus;* (ii) modified F2 medium (Guillard et al., 1962) without vitamins and containing 0.2 g L^{-1} of $Na_2HPO_4 \cdot 2\ H_2O$ and 0.75 g L^{-1} of $NaNO_3$ for *N. gaditana* and *N. oleoabundans;* (iii) modified M-8 culture medium

(Cuaresma et al., 2009) for *C. sorokiniana,* and (iv) modified Chu medium (Largeau et al., 1980) without citric acid and vitamins for both *B. braunii* strains.

All microalgal strains were maintained in linear phase cultures in shake flasks placed in a growth chamber at 25 ºC. The cultures were continuously illuminated at 110 μmol photons m^{-2} s^{-1} with fluorescent lamps and bubbled with air containing 2.5% (v/v) CO_2 as unique carbon source.

2.3.2 Cultivation conditions for the surfactant biodegradability test

For the biodegradability assays presented in Chapter 4, *S. obliquus* was maintained in a modified M-8 culture medium in which urea was replaced by KNO_3 (3 g L^{-1}) in order to avoid the presence of any other carbon source in the media apart from the surfactants.

The biodegradability of the surfactants was indirectly determined by following bacterial growth. Bacterial growth was studied at 1 and 10 CMC surfactant concentrations. The concentration of 1 CMC still allows for acceptable foam formation, however, possible inhibitory effects on bacterial cells are minimized. A high concentration of 10 CMC was chosen in order to increase the amount of carbon supplied. Thus, the change in turbidity is ensured to be detectable in case of a biodegradable surfactant while also possible surfactant inhibitory effects on bacterial growth could be detected. The experiments were performed in duplicates in 100 mL shake flasks. Surfactant solutions were prepared in modified M-8 media, with nitrate as nitrogen source, and they were sterilized by filtration. Each surfactant solution was inoculated with 10 mL of *S. obliquus* culture supernatant, which naturally contains microalgae-associated bacteria. The cultures were placed in an orbital shaker incubator (MaxQ 4000, Thermo Scientific, United States) at 37 ºC and 115 rpm. Bacterial growth was measured as the increment of optical density at 600 nm ($O.D._{600}^{culture}$) after 3 days (t_3), which was normalized by the initial optical density of each solution ($O.D._{600,t0}^{culture}$), as shown in Equation (1). In order to avoid errors related to turbidity changes in the surfactant solutions during the experimental time, blank cultures with the different surfactant solutions (not inoculated) were prepared ($O.D._{600}^{blank}$).

$$\boldsymbol{O.D.increase\ factor} = \frac{\left(O.D._{600,t3}^{culture} - O.D._{600,t3}^{blank}\right) - \left(O.D._{600,t0}^{culture} - O.D._{600,t0}^{blank}\right)}{\left(O.D._{600,t0}^{culture} - O.D._{600,t0}^{blank}\right)} \qquad (1)$$

2.3.3 Cultivation conditions for the assessment of the surfactants toxicity

In Chapter 4, the toxic effects of surfactants on the microalgal strains *C. sorokiniana*, *S. obliquus* and *N. gaditana* were tested at the following surfactant concentrations: 0.1, 0.5, 1, 2.5, 5, 10, 15, 25 and 50 CMC, provided the surfactant was still soluble at that concentration. Cultures were prepared in shake flasks containing 300 mL of surfactant solution prepared in the corresponding microalgal culture medium and inoculated with microalgal cells growing in linear phase. Microalgal cultures were prepared in triplicate under sterile conditions and biomass concentration was adapted in order to get an initial optical density of 0.8 at 750 nm. The cultures were not aerated in order to prevent foam formation. Instead of bubbling, the cultures were magnetically stirred at a speed of 200 rpm in order to maintain the homogeneity of the suspensions. Optical density at 750 nm (UV/ Visible spectrophotometer, Evolution 201, ThermoFisher, United States) and maximal photosynthetic efficiency of Photosystem II (AquaPen-C AP-C 100, Photon Systems Instruments, Germany) were measured daily for a minimum of 3 days.

Additional surfactant concentrations were tested in 6-well plates. Each condition was assessed in triplicate wells, while control cultures without the surfactant were performed in duplicate wells and one well was used for the blank (surfactant solution without microalgae). Each well had an initial volume of 7 mL. The plates were placed on an orbital shaker (MAXI HD, OVAN, Spain) set at 100 rpm and they were continuously illuminated at 100 µmol photons m^{-2} s^{-1} by fluorescent lamps from above.

2.3.4 Cultivation conditions to assess the biodegradability of BSA, Saponin and Tween 20 in *Scenedesmus obliquus* cultures

In Chapter 5, the effects of the surfactants BSA, Saponin and Tween 20 on the bacterial and microalgal populations were assessed in cultures of *S. obliquus.* The three surfactants were assessed at concentrations of 1, 5, 10, 25 and 50 CMC. Duplicate cultures were prepared in 100 mL flasks with 75 mL of modified M-8 culture medium, in which the original nitrogen source (i.e. urea) was replaced by KNO_3. The flasks were inoculated from a *S. obliquus* culture maintained in linear phase of growth. The inoculum volume was adjusted in order to get an initial

O.D.$_{750}$ of 0.2. The cultures were placed on an orbital shaker (MAXI HD, OVAN, Spain) set at 155 rpm and they were continuously illuminated at 110 µmol photons m^{-2} s^{-1} by fluorescent lamps from above. 1 mL culture samples were taken daily and centrifuged at 3000 g for 5 minutes (MiniSpin centrifuge, Eppendorf). Bacterial concentration was measured as the O.D.$_{600}$ of the corresponding supernatant of the culture broth samples. In turn, the resulting pellets were resuspended in 1 mL of fresh culture media and the microalgae concentration was measured as the O.D.$_{750}$ of these microalgal suspensions in order to reduce possible interferences due to bacteria presence.

Bacterial and microalgal growth was measured as the increment in O.D. during 2.8 days of cultivation, measured at 600 and 750 nm, respectively (ΔO.D.$_{600}$ and ΔO.D.$_{750}$). The parameter O.D.$_{600}$/O.D.$_{750}$ was calculated and used as an estimation of the bacteria to microalgae ratio. Moreover, 10 mL of culture were taken at the beginning and the end of the experiment in order to measure the dry weight of the cultures in duplicate and calculate the volumetric biomass productivities (see **Section 2.5.5** of this Chapter) achieved by each culture along the experiment.

2.3.5 Cultivation conditions to assess the effects of Pluronic F68 in liquid cultures of *Chlorella sorokiniana*

In Chapter 7, the effects of Pluronic F68 on the biochemical composition of *C. sorokiniana* were assessed in liquid cultures. *C. sorokiniana* was cultivated in 1-L flasks in 600 mL of M-8 culture medium. The conditions assessed included: (i) control cultures in complete M-8 culture medium, (ii) cultures in complete M-8 culture medium with Pluronic F68 at a concentration of 12.5 CMC, (iii) nitrogen-starved (N-starved) cultures in nitrogen-free M-8 culture medium, and (iv) N-starved cultures in nitrogen-free M-8 culture medium with Pluronic at a concentration of 12.5 CMC. The cultures were inoculated at an initial O.D.$_{750}$ of 0.5 from a *C. sorokiniana* culture growing in linear phase. The inoculum was obtained by centrifuging the corresponding culture volume at 3000 g for 5 minutes (Eppendorf 5702, Germany). The pellets were then added to their corresponding culture media. Each condition was tested in duplicate cultures which were incubated without bubbling to avoid foam formation. Instead, the cultures were magnetically stirred (Multi-Channel stirrer MS-52M, Jeio-Tech, Korea) in order to maintain the microalgal suspensions homogeneous.

2.3.6 Cultivation conditions to assess the effects of Pluronic F68 in foam-based cultures of *Chlorella sorokiniana*

In Chapter 7, the effects of Pluronic F68 on the biochemical composition of *C. sorokiniana* were also assessed in foam-based cultures. Cultivation was performed in the liquid foam photobioreactor (LF-PBR). The LF-PBR set-up is described in Chapter 6. The culture conditions assessed included: liquid base cases, foam-based controls, foam-based cultures in 3-fold concentrated culture medium, foam-based cultures under nitrogen starvation, foam-based cultures under saturating light intensities and foam-based cultures under nitrogen starvation and saturating light intensities. The particular operational conditions of the cultures are shown in **Table 2.3**. Besides, all cultures were performed in duplicate using two LF-PBR units and were bubbled with air containing 2.71% (v/v) CO_2 at a gas flow rate of 3000 mL min^{-1}, which resulted in a superficial gas flow rate of 2.5 10^{-3} m s^{-1}.

Table 2.3. Operational conditions of the *Chlorella sorokiniana* cultures performed in the LF-PBR. Culture conditions: liquid cultures (Liquid, base case), foam-based cultures in standard M-8 culture medium (Foam, control), foam-based cultures in 3-fold concentrated M-8 culture medium (Foam, 3-fold medium), foam-based culture in nitrogen-free M-8 culture medium (Foam, N-starved), foam-based culture in 3-fold concentrated M-8 culture medium under saturating light (Foam, saturating light) and foam-based culture in nitrogen-free M-8 culture medium under saturating light (Foam, N-starved + saturating light). For the preparation of the 3-fold concentrated M-8 culture medium all nutrient concentrations were 3-fold increased. For the preparation of N-free M-8 culture medium, all nutrients were added except for urea, which is the only nitrogen source used for this medium. Further detail regarding the two different illumination systems employed is provided in Chapter 6.

	Assays in the liquid foam-bed reactor					
	Liquid, base case	**Foam, control**	**Foam, 3-fold medium**	**Foam, N-starved**	**Foam, saturating light**	**Foam, N-starved + saturating light**
Culture medium	M-8	M-8	3-fold concentrated M-8	N-free M-8	3-fold concentrated M-8	N-free M-8
Surfactant and concentration	-	Pluronic F68, 12.5 CMC	Pluronic F68, 12.5 CMC	Pluronic F68, 12.5 CMC	Pluronic F68, 12.5 CMC	Pluronic F68, 12.5 CMC
Illumination system	Fluorescent lamps panel	Fluorescent lamps panel	Fluorescent lamps panel	Fluorescent lamps panel	Cylindrical LED system	Cylindrical LED system
Batch cultivation duration (d)	7	7	13	10	13	<2

2.3.7 Cultivation conditions for the assessment of fertilizer-based culture media in microplates

In Chapter 8, *Chlorella sorokiniana* was cultivated in 24-well plates (CELLSTAR®, Greiner Bio-One, Germany) to assess 10 different nitrogen fertilizers as nitrogen source (NF, **Table 2.2**, **Section 2.2.2** of this Chapter) and also to test culture media based on two NPK fertilizers (see **Section 2.2.3** of this Chapter). The NFs and NPK fertilizers were assessed in separate assays.

The NFs were used as a replacement of the original nitrogen source of the M-8 culture medium, urea. They were added to nitrogen-free M-8 at the corresponding concentration in order to approximate the original nitrogen concentration of the medium, 0.03 M. On the other hand, the NPK fertilizers 18-6-6 and 12-6-4 were prepared in distilled and tap water at the concentrations of 1 mL of NPK per liter of water. The NPK fertilizers compositions are detailed in **Section 2.2.3** of this Chapter.

In all cases, cultures were performed in duplicate and were inoculated with the corresponding volume of a *C. sorokiniana* culture growing in linear phase in order to obtain an initial optical density at 680 nm of 0.2. The inoculum was first centrifuged to remove the old medium, resuspended in distilled water in order to remove possible remaining nutrients and then centrifuged again prior being finally resuspended in the corresponding culture medium. For each fertilizer-based media, non-inoculated solutions were included and used as blank. The optical density of the blank solutions was subtracted to those of the corresponding cultures and the resulting values were used for the following calculations. In each well, the initial volume of the cultures and the fertilizer solutions was 2.5 mL. When multiple plates were used, the plates were placed next to each other in order to minimize the number of exterior wells, which are more susceptible to evaporation and were filled with distilled water. The plates were placed on an orbital shaker (MAXI HD, OVAN, Spain) set at 220 rpm. The orbital shaker was placed in a plant growth chamber (Bio Line Mod. VB 1014, Vötsch Industrietechnik, Germany) at 25 ºC and filled with 5% (v/v) CO_2-enriched air. The cultures were continuously illuminated at 335 µmol photons m^{-2} s^{-1}.

2.3.8 Cultivation conditions for the assessment of fertilizer-based culture media in flasks

In Chapter 8, *Chlorella sorokiniana* was also cultivated in flasks to assess different fertilizer-based culture media, including NF and NPK fertilizers, in separate assays. The NFs were used as a replacement of the original nitrogen source of the M-8 culture medium, urea. They were added to nitrogen-free M-8 at the corresponding concentration in order to approximate the original nitrogen concentration of the medium, 0.03 M. On the other hand, the NPK fertilizers 18-6-6 and 12-6-4 were prepared in tap water at the concentrations specified in the corresponding section of Results and Discussion. Unless otherwise indicated, all NPK-based culture media were supplemented with 0.1 mL L^{-1} of an agricultural micronutrient solution and 0.4 g L^{-1} of $MgSO_4$. When indicated, specific amounts of an agricultural P_2O_5 solution at 54% (w/w) were also added. The composition of the NPK fertilizers and the agricultural micronutrients solution used are detailed in **Section 2.2.3** of this Chapter.

In all cases, the different conditions were assessed in duplicate cultures in 500 mL flasks. When needed, the initial pH of the solution was adjusted to 6.8 prior inoculation using a 0.5M NaOH solution or a 0.1M HCl solution. The cultures were inoculated as described previously in the **Section 2.3.7** of this Chapter. Unless otherwise indicated, pH was measured daily and adjusted to 6.8 using 0.5M NaOH solution. The cultures were placed in a growth chamber at 25 ºC, continuously illuminated at 110 µmol photons m^{-2} s^{-1} with fluorescent lamps and bubbled with air containing 2.5% (v/v) CO_2 as unique carbon source.

For the experiments performed in batch mode, the initial $O.D._{680}$ of the cultures is detailed in the corresponding section of Results and Discussion. The cultures assessed in repeated batch were maintained at a $O.D._{680}$ range between 1.2 and 2.5.

2.3.9 Cultivation conditions for cultures with Pluronic F68 in NPK-based culture media

In Chapter 8, the adequacy of an NPK-based culture media in combination with the surfactant Pluronic F68 was assessed in liquid cultures of *C. sorokiniana.* The different media assessed included: modified M-8 culture medium, modified M-8 culture medium with the addition of Pluronic F68, NPK-based culture medium and NPK-based culture medium with the addition of Pluronic F68.

The M-8 culture medium was modified in order to avoid nutrients waste along the repeated-batch cultivation, since the original nutrients concentrations of this medium are committed to high cell density cultures operated in continuous mode in photobioreactors. The concentration of nitrogen, iron, calcium and magnesium were reduced down to the following: [N]= 53 mM, [Fe]= 0.1 mM, [Ca]= 0.08 mM and [Mg]= 0.8 mM. The NPK-based media were prepared with the NPK fertilizer 8-6-6 (Agralia Fertilizantes S.L., Spain), which contained urea as the main nitrogen source. The NPK was added at the concentration of 4 mL per liter of culture in order to approximate the nitrogen concentration of the modified M-8 culture medium. Due to the use of distilled water to prepare the media, the NPK-based media were supplemented in magnesium and calcium by adding $MgSO_4{\cdot}7H_2O$ and $CaCl_2$ at final media concentrations of 0.8 and 0.08 mM, respectively, since those were the final concentrations of these nutrients in the M-8 media. Besides, in order to avoid growth limitation due to lack of trace elements in the commercial NPK, an agricultural solution of micronutrients was also used (Agralia AG Complex, Agralia Fertilizantes S.L., Spain). The composition of this solution is detailed in **Section 2.2.3** of this Chapter. Finally, media supplemented with the surfactant Pluronic F68 were prepared at a concentration of 12.5 CMC (Critical Micelle Concentration = 0.334 g L^{-1})

All the conditions were assessed in duplicate cultures in 500 mL flasks. The cultures containing the different media were incubated in the algal room where temperature was automatically controlled at 25 ºC and light intensity was supplied by white fluorescent tubes (Philips 30W/33) at a light intensity of 100 μmol photons m^{-2} s^{-1} at the flasks surface. Mechanical agitation was provided by an orbital shaker (MAXI HD, OVAN, Spain) in order to maintain the different cultures homogeneous rather than aeration with CO_2-enriched air due to the imminent foam formation related to the surfactant presence in some of the cultures. The experiment was performed in repeated-batch cultivation for 42 days, along which the $O.D._{750}$ of the cultures was maintained at values in the range of 0.3-2.5 by punctual dilutions. The first and the last growing cycle were used as representative from non-adapted and adapted cultures, respectively. Biomass concentration was measured as $O.D._{750}$ and dry weight and, when indicated, the values were normalized to the initial biomass concentration. The pH of the cultures was measured daily and maintained at 7.0 ± 0.5.

2.3.10 Incubation conditions for the assessment of the extractant capability of Pluronic F68

In Chapter 9, the capability of Pluronic F68 to extract fatty acids from *C. sorokiniana* cells was evaluated at the following surfactant concentrations: 0, 12.5, 31.25, 62.5 and 125 CMC (CMC_{F68}= 0.334 g L^{-1}). In order to get multiple samples of 5 mg of freeze dried biomass, aliquots of the corresponding volume were taken from a *C. sorokiniana* culture grown under nitrogen deprivation for 3 days, centrifuged at 3000 g for 5 minutes (Eppendorf 5702, Germany) and washed with distilled water 3 times prior being freeze dried. The surfactant solutions were prepared in distilled water and 5 mL of each solution were added to each freeze dried algal biomass sample. The freeze dried biomass was resuspended in the surfactant solution by vortexing and the tubes were tightly closed before incubation. The incubation of the samples was performed at room temperature in an orbital shaker (MAXI HD, OVAN, Spain) set at 100 rpm for 0, 1, 2, and 4 days. In order to calculate the possible fatty acids release under the different conditions assessed, two different treatments were performed to replicate samples. First, to determine the fatty acid content that remained in the biomass after the incubation, the samples were centrifuged at 3000 g for 5 minutes (Eppendorf 5702, Germany), and the resulting pellets were freeze dried for the subsequent lipids extraction and fatty acids analysis. Besides, to analyze the possible fatty acids released to the supernatant during incubation, not centrifuged samples (containing the biomass and the supernatant) were freeze dried before the lipid extraction and fatty acids determination. Samples no subjected to the incubation process (Control) and samples incubated with no surfactant (0 CMC samples) were used as controls. In all cases, duplicate samples were analyzed. The experimental set-up is schematically described in **Figure 9.4** (Chapter 9).

2.4 Foam-related assays

2.4.1 Foamability test

In Chapter 3, natural foamability was assessed for the 6 algal strains in their corresponding culture media, by using culture samples from linear and stationary phase cultures. Moreover, BSA-mediated foamability of the different culture media without microalgae was tested at different BSA concentrations, ranging from 0.02 to 0.1 g L^{-1}, in order to evaluate the influence of culture media salinity and to identify the optimal BSA concentration for the assays with algal samples supplemented with BSA. Besides, the effect of biomass concentration on foamability was also evaluated in the BSA-mediated foamability assays with algal samples and for that 3-fold concentrated biomass samples were used and prepared by centrifugation at 3000 g for 5 minutes (Eppendorf 5702, Germany). Sample pH was adjusted to 7.0 before performing the tests.

The foam production system (**Fig. 2.2**) consisted of 1-L glass graduated cylinder with 2 air stone-diffusers placed at the bottom. 200 mL of the corresponding suspension (algal broth, culture media supplemented with BSA or 3-fold concentrated algal broth supplemented with BSA) were sparged with small air bubbles at an air flow of 0.9 L min^{-1} and the height level of the foam was monitored every 15 seconds for a maximum of 15 minutes. Foam production speed is expressed in mm s^{-1}, and refers to the foam height produced after the first 30 seconds of bubbling.

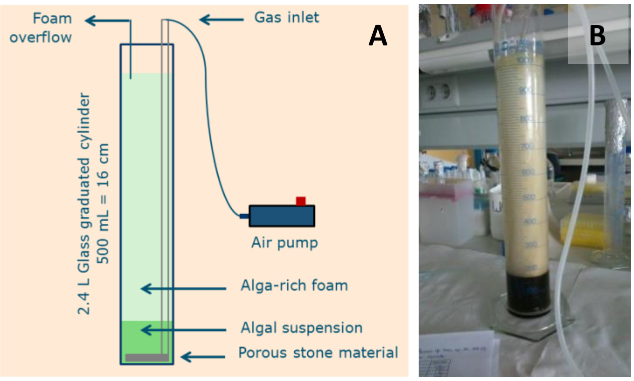


Figure 2.2. (A) Schematical description of the foam production system used for the foamability tests. (B) Picture of a foamability test performed for a suspension of *Phaeodactylum tricornutum*.

2.4.2 Microalgal partitioning test

The microalgal partitioning refers to the distribution of the algal biomass between the liquid and the foam phases when foam is produced by sparging a liquid culture sample.

For the microalgal partitioning tests presented in Chapter 3, samples were taken from linear phase cultures and assessed at different biomass concentrations (1.5-2 g L^{-1} and 8-10 g L^{-1}). In case pre-concentration was needed the samples were centrifuged at 3000 g for 5 minutes. 0.1 g L^{-1} BSA was added to 100 mL of algal suspension and the pH was adjusted to 7.0. The suspension was placed in a 250 mL flask and sparged trough 2 air stone-diffusers. The first foam produced was collected by a funnel in a sample tube (**Fig. 2.3**). Time of bubbling was the minimum required to obtain 10 mL of liquid sample in the test tube after the foam collapsed. That time was shorter than one minute at all events.

Figure 2.3. Collection in a test tube of the liquid released from the foam after a BSA-added microalgal suspension was bubbled with air during the microalgal partitioning test.

Biomass concentration of the liquid released was measured in terms of dry weight ($C_{iquid\ from\ the\ foam}$). Microalgal partitioning was calculated according to Equation (2) by comparison with the initial biomass concentration in the liquid suspension ($C_{nitial\ liquid\ suspension}$). For the definition of the microalgal partitioning the initial biomass concentration is considered instead of the concentration in the bulk liquid since the bulk liquid biomass concentration is continuously changing with increasing foam volume.

$$Microalgal\ partitioning\ (-) = \frac{c_{liquid\ from\ the\ foam}(g\ \ L^{-1})}{c_{initial\ liquid\ suspension}(g\ \ L^{-1})} \qquad (2)$$

For the algal partitioning assays presented in Chapter 4, *C. sorokiniana* was grown on 3 times concentrated M-8 media, in which the N and P content were further modified to enable high biomass densities compared to the M-8 recipe described previously (Kliphuis et al., 2010). The growth medium for *S. obliquus* was based on the medium from Breuer and co-workers (2014) but further modified in order to reach higher biomass densities. Both microalgae were grown in 250-300 mL shake flasks placed in an orbital shaker in an incubator. The incubator settings were the following for *C. sorokiniana*: 37 °C, 454 µmol photons $m^{-2}s^{-1}$, 120 rpm and 4% CO_2, and for *S. obliquus*: 25 °C, 120 µmol $m^{-2}\ s^{-1}$, 80-100 rpm and 2.5% (v/v) CO_2.

For the determination of the microalgal partitioning in Chapter 4, microalgae-surfactant mixtures were prepared by adding the surfactant to the microalgal culture (*C. sorokiniana* or *S. obliquus*). This mixture was foamed in a 2.5 L glass tank (20.5 x 3 x 40 cm, width x depth x height). Silicon tubing (internal diameter of 4 mm, wall thickness of 2 mm) punctured by a 0.45 µm needle at 1.4 cm intervals served as gas distributor. This tubing was placed one cm above the bottom of the glass tank over the complete width. A gas flow rate of 1000 mL min^{-1} was supplied to the surfactant solutions containing microalgae to allow foam formation. The volume of the algae suspension was 200 mL for *C. sorokiniana* experiments, and 100 mL for *S. obliquus.* The difference in the volumes was due to the fast settling of *S. obliquus* cells underneath the gas distributor. For this reason, the volume underneath the gas distributor was filled with 100 mL of solidified Agarose gel (5%), and only 100 mL algae suspension was used to keep the same liquid height before foaming. The surfactant concentration was 5 CMC in order to ensure sufficient foam formation, except for Saponin where 5 CMC did not generate enough foam for the measurements; thus, 10 CMC was used. BSA was tested at a concentration of 1.75 g L^{-1}, which represents the concentration applied previously in a liquid foam-bed photobioreactor (Janoska et al., 2017). For each surfactant, duplicate foaming experiments were performed.

The experiments with *C. sorokiniana* were started at an optical density at 750 nm ($O.D._{750}$) of 15 to 20, while the experiments with *S. obliquus* were started with an $O.D._{750}$ of 2. Foam was allowed to rise in the glass tank, and the foam flowing out on top was collected. In each experiment, four different foam samples of 150 mL were taken, provided sufficient foam was formed. The first 150 mL of foam was discarded and the remaining three foam fractions were allowed to collapse in cups. A sample was taken from the collapsed foam and the O.D. $_{750}$ was measured in duplicate. Afterwards, these O.D.$_{750}$ values were averaged for the three different foam fractions, and the two duplicate experiments. The optical density was measured at 750 nm in a spectrophotometer (Hach Lange DR6000, Germany). As SDS solutions were opaque the cell concentration was determined by cell counting: microscopic cell count using counting chamber and automatic cell counting with Beckman Coulter Multisizer 3 employing a 50 μm aperture tube.

2.4.3 Algal hydrophobicity test

Hydrophobicity of microalgae was measured by using a modified adherence-to-hydrocarbon method previously reported (Garg et al., 2012) which is based on the distribution ratio of the cells between hydrophilic and hydrophobic liquids.

In Chapter 3, culture samples were collected along the growth curve for all the microalgae, including lag, linear and stationary phase, and at a minimum of 6 different time points. The initial optical density of the samples at 680 nm was adjusted to 1.2 by dilution with the corresponding culture medium and the pH was adjusted to 7.0. Moreover, for linear phase samples, BSA-added samples were also tested in order to determine the effect of this surfactant on the algal hydrophobicity. To avoid the interference of BSA turbidity, the values for BSA-added samples were corrected with the corresponding cell-free culture media controls. All the samples were vigorously mixed with hexane during 1 minute and after that time separation of the 2 phases was allowed during a settling time of 2 minutes (**Fig. 2.4**).

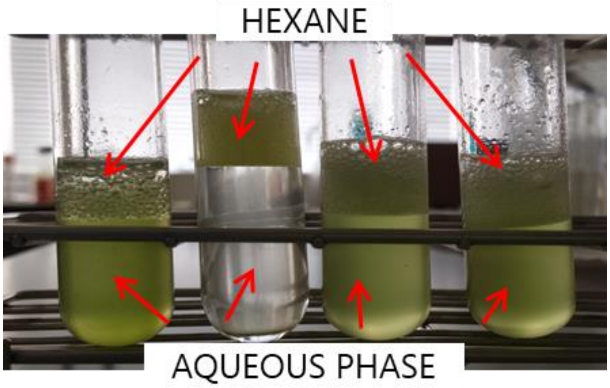


Figure 2.4. Microalgal samples after being subjected to the algal hydrophobicity test. Hexane (top) and aqueous (bottom) phases are indicated by red arrows.

The algal hydrophobicity (H) was calculated as the extractability of hexane according to Equation (3):

$$H\ (\%) = \left(\frac{O.D._0 - O.D._w}{O.D._0}\right) \times 100 \qquad (3)$$

where $O.D._o$ is the initial optical density of the microalgal suspension at 680 nm and $O.D._w$ is that of the aqueous phase after being settled for 2 min.

Moreover, the effect of Pluronic F68 on microalgal hydrophobicity was also measured for different microalgal strains (Chapter 7). For that, the algal samples were obtained from microalgal cultures growing in linear phase, and the surfactant was added at a concentration of 1 CMC.

2.4.4 Foam stability test

Foam lifetime $t_{2/3}$, the time in which foam height decreased two thirds of the initial foam height, was defined as a parameter to describe foam stability. Additionally, foam collapse speed was calculated. This is the foam column height decrease produced during the first 2 minutes and expressed in mm s^{-1}.

The high concentrated algal samples (8-10 g L^{-1}) employed to determine microalgal partitioning were used for the foam stability tests. A sample volume of 100 mL containing 0.1 g L^{-1} of BSA was placed in a 1-L plastic graduated cylinder. Algal suspension was sparged with air through two small stone-diffusers placed at the bottom until foam reached the top of the system (**Fig. 2.5**).

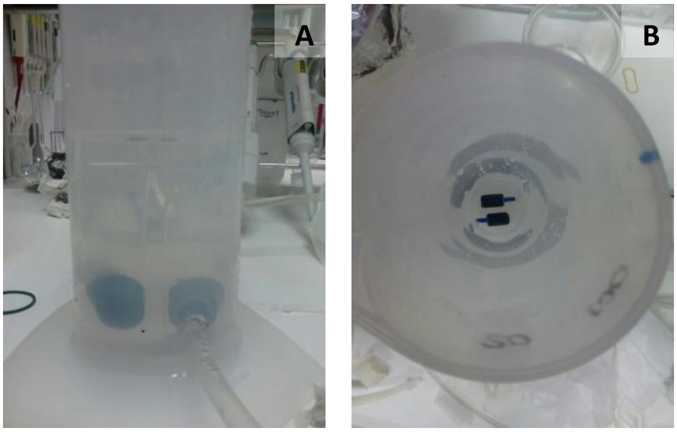


Figure 2.5. (A) Front and (B) top views of the experimental set-up used to perform the foam stability tests. The two small stone-diffusers placed at the bottom of the graduated cylinder are shown.

Foam level decrease was monitored every 15 seconds until the foam collapsed completely. The top of the foam column was often not flat during foam collapse. Thus, the minimal height that was completely covered with foam was considered when measuring the foam column height. Foam stability test was performed only for the samples whose corresponding foams achieved the top of the cylinder.

2.4.5 Surfactant foaming properties

In Chapter 4, the foaming properties of the different surfactant solutions were determined by an automatized foaming device (FoamScan, Teclis- IT Concept, Logessaigne, France), as described previously (Janoska et al., 2017). Foam stability is measured in terms of the time elapsed until half of the foam volume had collapsed, and this will be referred to as the foam half-life. The liquid holdup of the foam represents the amount of liquid incorporated in the foam at the moment when the foam reached its desired volume and the gas supply stopped. All experiments were performed at a temperature between 28 and 30 °C. The gas flow rate employed was 400 cm^3 min^{-1}, resulting in 2.4 mm s^{-1} superficial gas velocity. Two different concentrations were used for each surfactant: 1 and 5 CMC. BSA was measured at 1 g L^{-1} and 5 g L^{-1} at higher temperatures of 37 °C (Janoska et al., 2017). The experiments were performed in duplicates.

The bubble size was analyzed from pictures taken 30 seconds after the gas flow had stopped, at a height of 8 cm above the gas distributor. The mean bubble size and the standard deviation were calculated by an image analysis software

(Foamscan) for each measurement, and the standard deviations and the average of the means of the two independent measurements were calculated.

The stability of the surfactants during repetitive foaming was assessed by comparing the up-flow speed of foams formed by fresh surfactant solution and the same solution after 20 continual cycles of foam production and subsequent deconstruction. Foam was produced from 200 mL surfactant solution in a 1-L glass graduated cylinder by distributing air through two small-bubble diffusers at a superficial velocity of 3.68 mm s^{-1}. Surfactant solutions were prepared in M-8 culture medium adjusted to pH 6.8. Foam up-flow speeds were calculated for a 30 seconds foaming time by measuring the foam level. Each surfactant test was performed in a maximum of 10 hours to prevent losses of foaming properties due to other causes (e.g. biodegradability, light sensitivity). The minimum surfactant concentration resulting in maximal foam-up flow speed (C_{min}) was determined and 1.1 times C_{min} was used in the stability tests. C_{min} values (expressed in terms of CMC) found for the different surfactants were: BSA, 5; CTAB, 0.5; Pluronic F68, 5; Pluronic P84, 4.25; Saponin, 0.5; SDS, 0.08; Tergitol NP9, 1; Tergitol TMN6, 1; Triton X-100, 0.75, and Tween 20, 0.5.

2.4.6 Assessment of the effects of surfactant biodegradation on foaming capacity

In Chapter 5, the effects of Saponin biodegradation on the foaming capacity of this surfactant was tested. For this experiment, the *S. obliquus*-associated bacteria were isolated (see **Section 2.1.2** of this Chapter) and used. Prior to the experiment, both the surfactant and the bacterial culture medium concentration were optimized. For the determination of the optimal Saponin concentration, the foamability of Saponin solutions was measured for Saponin solutions at 0.5, 1.0, 1.5 and 2.0 CMC prepared in 100 mL of Luria-Bertani (LB) medium using the method described in **Section 2.4.1** of this Chapter. The minimum Saponin concentration at which the maximum foamability was achieved was used for the experiment. On the other hand, the optimal concentration of LB medium was determined. For that, cultures of *S. obliquus*-associated bacteria were prepared in 1-L flasks in LB medium diluted 2, 5, 10 and 20-fold. The volume of inoculum was selected to get an initial optical density at 600 nm ($O.D._{600}$) of approximately 0.1. The cultures were prepared in duplicate and incubated in and orbital shaker incubator (MaxQ 4000, Thermo Scientific, United States) at 37 °C and 115 rpm. Bacterial growth was measured as $O.D._{600}$ and the optimal LB medium concentration was determined as the lowest concentration that resulted in bacterial growth cease during the experimental time (i.e. 72 h).

According to the aforementioned tests, the experiment was performed using Saponin at 2 CMC and LB/20 culture medium. The conditions assessed included: non-inoculated bacterial culture medium, non-inoculated Saponin solutions, Saponin-free bacterial cultures and bacterial cultures supplemented with Saponin. The cultures were prepared in duplicate in 1-L flasks and incubated in an orbital shaker incubator (MaxQ 4000, Thermo Scientific, United States) at 37 °C and 115 rpm for 11 days. Samples of 105 mL were taken daily. Bacterial growth was measured as $O.D._{600}$ and the foamability of the cultures was assessed by measuring the foamability as described in **Section 2.4.1** of this Chapter using 100 mL of the culture sample.

2.4.7 Effect of temperature on the foamability of Pluronic F68

The effect of temperature on the foamability of Pluronic F68 was determined by measuring the foamability of Pluronic F68 solutions prepared at 12.5 CMC in M-8 culture medium incubated at 18 °C (i.e. room temperature) and 37 °C for different periods of time (0, 4, 8 and 16 hours). The surfactants solutions were prepared in 1-L flasks and incubated in an orbital shaker (IKA KS 4000 ic control) for incubation at 18 °C and MaxQ 4000, Thermo Scientific, United States for incubation at 37 °C) at a speed of 150 rpm. The foam production speed was measured in a liquid foam photobioreactor (LF-PBR), which was graduated for the experiment. The solutions were bubbled with air and the foam production speed was measured as the maximum height achieved by the foam column after 30 seconds. The maximum height considered was the maximum height that was completely covered by foam after 30 seconds of foaming.

2.5 Physical determinations

2.5.1 Dry weight

Microalgae samples were filtered through pre-washed, pre-dried and pre-weighed filters (glass microfiber filters with a pore size of 0.7 μm) (Filter Lab MFV5, Filtros Anoia S.L., Spain). Filters were then dried at 80 ºC during at least 16 h and cooled down in a desiccator for at least 2 h. The filter weight was determined on a 0.01 mg precision balance (Sartorius CP225D, Sartorius AG, Germany). The dry weight concentration, expressed as g L^{-1}, was calculated by differential weight.

2.5.2 Optical density

The optical density (O.D.) of the cultures was measured in a UV-Vis spectrophotometer (Evolution 201, ThermoFisher, United States) at 600 nm to determine bacterial growth and at 680 and/or 750 nm to measure microalgal growth.

2.5.3 Cell number

Microalgal cell concentration was measured by counting cells in a Neubauer chamber by observation under an optical microscope (DM 1000, Leica Microsystems, Germany). A Neubauer chamber is composed by a thick slide with 2 counting grids and a glass cover. The Neubauer chamber's grid is formed by nine 1 mm^2 squares, from which the ones in the corners were used for microalgal cells counting. When the glass cover is placed on top of the slide, the distance between them is 0.1 mm, resulting in a volume of 0.1 mm^3 (i.e. 0.1 mL) for each square. The cells contained in at least two squares of each grid were counted, and the average cell number per square ($L_{average}$) was used to calculate the cell concentration in the sample according to Equation (4). If needed, the culture sample was diluted in the corresponding culture medium prior counting and the dilution factor (*d.f.*) was considered for the calculations.

$$Cell\ concentration\ (cells\ mL^{-1}) = L_{average} \cdot 10^4 \cdot d.f. \tag{4}$$

2.5.4 Growth rate

Microalgal growth rate (μ) was calculated using Equation (5), where C_{tx} is the biomass concentration at a given experimental time and C_{tx-1} is the initial biomass concentration. The growth rates were calculated in the linear phase of growth (from $t_{x-1}$to t_x). The time period employed was specified in the corresponding section of Results and Discussion for each experiment.

$$\mu = Ln\frac{\left(C_{tx}/C_{tx-1}\right)}{(t_x - t_{x-1})} \tag{5}$$

Unless otherwise indicated, dry weight values were used for the calculation of the growth rates.

2.5.5 Volumetric biomass productivity

Microalgal biomass productivities were calculated as the increase in volumetric biomass dry weight occurred for a certain period of time and it was expressed in grams of biomass produced per liter of culture per day (g L^{-1} d^{-1}).

The volumetric productivities of the microalgal metabolites (i.e. carbohydrates, fatty acids, carotenoids) were calculated similarly and expressed in the corresponding units.

2.5.6 Normalized O.D.$_{680}$ rate and ΔO.D.$_{680}$

Due to volume limitation, optical density was the only parameter that was measured in the cultures performed in 24-well plates. Optical density at 680 nm was employed to calculate the O.D.$_{680}$ rate (h^{-1}), which was used as an indicator of growth rate. O.D.$_{680}$ rate refers to the increase in optical density per time and it was calculated for periods of time along which linear growth was observed. These particular time periods are specified in the corresponding section in Results and Discussion. After being corrected by subtracting the optical density of their corresponding blanks, the values were normalized considering a linear growth and adjusting the initial optical density value of each culture to 0.1. The normalization was performed considering a linear growth as explained in **Section 2.5.2** of this Chapter. The normalized O.D.$_{680}$ rate was then calculated for each time period (from t_{x-1} to t_x) according to Equation (6) using the normalized O.D.$_{680}$ values:

$$Normalized\ O.D._{rate} = \frac{(Normalized\ O.D._{tx} - Normalized\ O.D._{tx-1})}{(t_x - t_{x-1})} \tag{6}$$

Besides, microalgal growth in 24-well plates was also expressed as ΔO.D.$_{680}$, which refers to the increment of optical density at 680 nm occurred in a specific period of time. For its calculation, the optical densities of the corresponding blanks were previously subtracted.

2.5.7 Biomass yield of the NPK fertilizers

The biomass yield of a NPK fertilizer is expressed in Chapter 8 as $Y_{X/N}$, and refers to the amount of biomass produced per milliliter of NPK. The biomass yield of a NPK fertilizer was calculated according to Equation (7).:

$$Y_{X/_N} = \frac{(V*X_1 - V*X_2)}{V*C_{NPK}} \tag{7}$$

Where V is the volume of culture, X is the biomass concentration expressed in grams per liter, and C_{NPK} is the concentration of NPK added to the culture, expressed in mL of NPK per liter.

2.5.8 Gram staining

For a general bacterial classification, *S. obliquus*-associated bacteria were Gram stained. The Gram stain differentiates bacteria into two fundamental groups depending on whether they retain the initial crystal violet stain (Gram-positive) or not (Gram-negative). This staining response is based on the different chemical structure of the cell walls of these bacterial varieties. Gram-positive bacteria have a thick cell wall composed by peptidoglycan and secondary polymers, whereas Gram-negative ones have a thin layer of peptidoglycan and an outer lipid-protein membrane. For the Gram staining, the bacterial suspension sample was fixed by adding few drops of methanol and attached to a glass microscope slide by gentle heating. A few drops of crystal violet stain (Crystal violet oxalate, Scharlau, Spain) were added and the sample was allowed to stand for one minute. Then, the sample was gently washed with distilled water and a few drops of Lugol solution (Lugol's solution, Scharlau, Spain) were added. After one minute the sample was gently washed with distilled water. A few drops of acetone were added to the sample in order to decolorize the Gram-negative bacteria. After 20 seconds, the sample was washed with distilled water and a few drops of Safranin solution

(Safranin O, Scharlau, Spain) were added to the sample to dye the Gram-negative bacteria. After washing the sample with distilled water, the dyed sample was observed under the optical microscope using the oil immersion objective lens.

2.5.9 Inverted microscope

Surfactant-added microalgal cultures were occasionally observed under an inverted microscope (Olympus cooperation IX71S8F-3, SN: 8F15047, Camera: XC30, Software: cellsens standard 1.14) in order to distinguish morphological changes in the microalgal cells and large increases in bacterial populations (Chapter 4).

2.5.10 Scanning Electron Microscope (SEM)

Biomass cells were fixated with a solution containing 1% glutaraldehyde, 2% paraformaldehyde and 0.1 M cacodylate buffer (0.1N). The resulting pellets were repeatedly washed with cacodylate buffer 0.1M and later they were dried by following the critical point drying principle. It is a method of dehydrating biological tissues while preserving the surface details. In this case, the water contained in the biological tissues was replaced by acetone and later on by liquid CO_2 which could be evaporated at approximately 35 ºC and at a pressure of 1200 psi. When the temperature rose above the critical temperature for liquid CO_2 (35 ºC) it changes to vapor without any change of density and therefore without surface tension effects which may distorts morphology and ultra-structures of cells. After that, samples were coated. Sputter coating consisted in applying an ultra-thin coating of an electrically conducting metal mixture containing gold and platinum. Creating a conductive layer of metal on the sample inhibits charging, reduces thermal damage and improves the secondary electron signal required for topographic examination in the SEM (General Research Services of University of Sevilla).

2.6 Analytical determinations

2.6.1 Pluronic F68 determination

Pluronic F68 concentration was measured in liquid culture media and microalgal suspensions by using a colorimetric method based on the formation of a dark blue complex between the surfactant and cobalt thiocyanate (Chung et al., 2011). To determine the Pluronic F68 concentration, 25 µL of trichloroacetic acid and 250 µL of methanol were added to a volume of 250 µL of sample. For samples with a Pluronic F68 concentration of approximately 10 CMC, 25 µL of sample was taken and a dilution factor of 10 was considered for the calculations. The solution was mixed by vortex for 30 seconds and centrifuged for 5 minutes at 3000 g. The supernatant was then transferred to a new tube with 250 µL of reagent and 250 µL of ethylacetate. The reagent is an aqueous solution of 20% (w/v) ammonium thiocyanate and 3% (w/v) cobalt nitrate hexahydrate. The suspension was mixed by vortex for 30 seconds and centrifuged at 3000 g for 5 minutes (MiniSpin centrifuge, Eppendorf). The two upper layers were carefully removed by aspiration and the pellet and the tube walls were washed with 1 mL of ethyl acetate 3 times. The pellet was dried in the fume hood for a minimum of 15 minutes and dissolved in 1 mL of acetone by vortex. The optical density of the samples was measured at 624 nm and the Pluronic F68 concentration was calculated by using a correlation curve prepared with standard solutions. For the correlation curve, a stock solution of Pluronic F68 (1500 mg L^{-1}) was prepared in M-8 culture medium, and the optical density of diluted solutions (50, 100, 500, 1000 and 1500 mg/L) at 624 nm ($O.D._{624}$) was measured. The resulted Equation (8) used for the quantification of Pluronic F68 is shown below. All the samples were analyzed at least in triplicate and the values presented correspond to the average value of the different replicates.

$$[Pluronic\ F68](CMC) = \frac{1213.59 \cdot O.D._{624}}{Sample\ volume\ (\mu L) \cdot Acetone\ volume\ (mL)} \tag{8}$$

2.6.2 Extraction and quantification of photosynthetic pigments

A determined volume of culture (lower than 1 mL in all cases) was centrifuged for 8 minutes at 3000 g. The pellet was resuspended in 5 mL of pure methanol and placed in an ultrasound bath for 5 minutes to be disaggregated. The suspension was kept in a thermoblock for 15 min at 60 °C and next incubated at 0 °C in ice. The suspension was then centrifuged for 8 minutes at 3000 g. Chlorophyll (Chl_{total}) and total carotenoids ($Carot_{total}$) concentrations were determined in the corresponding supernatant according to the equations below (Lichtenthaler, 1987).

$$Chl_a = (16.72 \cdot A_{665.2} - 9.16 \cdot A_{652.4}) \cdot dilution\ factor \tag{9}$$

$$Chl_b = (34.09 \cdot A_{652.4} - 15.28 \cdot A_{665.2}) \cdot dilution\ factor \tag{10}$$

$$Chl_{total} = Chl_a + Chl_b \tag{11}$$

$$Carot_{total} = \frac{dilution\ factor \cdot 1000 \cdot A_{470} - 1.63 \cdot Chl_a - 104.96 \cdot Chl_b}{221} \tag{12}$$

2.6.3 Lutein content

The specific carotenoids present in the methanolic extracts were separated by HPLC (TermoQuest, Thermo Separation Products equipped with diode-array detector). In the mobile phase, ethyl acetate was used as solvent A and a mixture of acetonitrile and water (9:1 v/v) was used as solvent B. The method is completely described in the study by Casal and co-workers (2011). The lutein content was quantified using a lutein standard (DHI-Water, and Environment, Hørsholm, Denmark).

2.6.4 Carbohydrates content

The carbohydrates content of *C. sorokiniana* biomass (Chapter 7) was determined using a colorimetric method based on the Dubois principle (Dubois et al., 1956; Sadasivan and Manickam, 2008). An amount of freeze dried biomass ranging from 3 to 10 mg was subjected to an acid hydrolysis. For that, 2.5 M HCl was added in a proportion of 0.5 mL per mg of biomass and the samples were incubated at 100 °C for 1.5 hours in a water bath. A volume of 2.5 M NaOH equal to that added of the acid solution was added to the samples in order to neutralize the acid. A volume of 500 µL of phenol and 2.5 mL of sulfuric acid were added to the samples which were then incubated at 35 °C for 30 minutes. The optical density of the samples at 483 nm was measured spectrophotometrically (Evolution 201, ThermoFisher, United States) and the carbohydrates content of the samples was calculated using a calibration curve prepared with glucose at concentrations in the range of 0-0.1 mg L^{-1}.

2.6.5 Fatty acid content and specific fatty acids profile

The total amount of fatty acids (FA) in the microalgal biomass (pellet) and in the supernatant (i.e. FA content in the total broth - FA content in the pellet) was measured and presented in Chapter 7 and 9. Lipids were extracted from freeze-dried biomass samples of 10-50 mg using 1:1:0.6 methanol/chloroform/water mixture and vortex for 20 seconds. Samples were centrifuged at 3000 g for 5 minutes (Centrifuge 5702, Eppendorf, Germany) and the lower layer was transferred to a glass vial. This step was repeated twice. The solvent was evaporated with a nitrogen stream at room temperature. The transesterification was performed by incubating the samples with 20:1 methanol-acetyl chloride (transesterification reagent) and pentadecanoic acid (internal standard) at 80 °C for one hour in a dry bath (Dry bath FB15103, Fisher Scientific). Fatty acid methyl esters were transferred to 1 mL of hexane and stored at -20 °C until their analysis by gas chromatography.

2.6.6 Determination and quantification of fatty acid methyl esters (FAMEs)

The hexane solutions were analyzed by gas chromatograph (6890N, Agilent) for the determination and quantification of the microalgal fatty acids. The gas chromatograph included an automatic sampler (7683 B, Agilent) and a flame ionization detector (FID) with a capillary column (Omegawax 320, supelco) with length, width and internal diameter dimensions of 30 m, 0.32 mm and 0.25 µm, respectively. Helium was used as carrier gas at a flow rate of 20 mL min^{-1}.

The temperature gradient used to obtain defined peaks in the chromatograms is presented in **Table 2.4**. The FID detector temperature was set at 260 °C. Split injector mode and injection volume of 1 µL were used.

The specific fatty acids present in the microalgal samples were identified and quantified by comparing their retention times with those of a mix of commercial standards with known concentrations (Supelco 37 FAME Mix, ref CRM47885). The commercial standards mix had a purity of 98.7-99.9% and, as the samples, it was diluted in hexane. The conversion factors for the fatty acids were obtained from the resulting chromatograms of the standards mix.

Table 2.4. Temperature gradient employed for the fatty acids determination.

Oven ramp	Slope (ºC min^{-1})	Temperature (ºC)	Retention time (min)	Runtime (min)
0	-	90	0	0
1	10	140	0	5
2	5	165	2	12
3	1	190	2	39
4	5	220	15	60

2.6.7 Proteins content

The protein content of the microalgal biomass (Chapter 7) was estimated from the nitrogen content measured by elemental composition analysis, using 4.5 as nitrogen-to-protein conversion factor (NTP) (López et al. 2010). Elemental analysis (determination of carbon, hydrogen and nitrogen contents) was performed by a calorimetric method by the General Research Services of University of Huelva. The amount of nitrogen in the samples was determined according to Gnaiger and Bitterlich (1984).

2.6.8 Moisture and ash content

In order to avoid underestimation of the metabolites content, the biochemical composition of the biomass (i.e. content of pigments, carbohydrates, fatty acids and proteins) was corrected considering the ash content of the biomass samples. Ash content is the inorganic residue remaining after the water and the organic matter have been removed. For ash content determination, ceramic cubicles were first weighed ($W_{cubicle}$) after being incubated at 550 °C for 2 hours and cooled down for 30 minutes in a desiccator. A minimum of 10 mg of freeze dried biomass was weighed in ceramic cubicles ($W_{samples}$) in triplicate and incubated for 3.5 hours at 550 °C. After being cooled down in a desiccator for 30 minutes, the cubicles containing the ashes were weighed ($W_{residue}$) and the ash content was calculated using Equation (13).

$$Ash\ content\ (\%) = \frac{(W_{residue} - W_{cubicle})}{W_{sample}} \tag{13}$$

Similarly, the moisture content of the biomass samples was measured in order to avoid underestimation of the main metabolites content. For that, freeze dried biomass samples of a minimum of 10 mg were weighed in triplicate in Petri dishes. The samples were equilibrated at room temperature for a minimum of 2 hours and weighed (W_1= moist samples). The samples were then incubated at 100 °C for 24 hours and weighed after being cooled down in a desiccator for 20 minutes (W_2= dried samples). Residual moisture content ($f_{moisture}$) is the difference of weight as shown in Equation (14).

$$f_{moisture} = W_1 - W_2 \tag{14}$$

All metabolites content values presented in this Thesis were corrected according to the corresponding ash and moisture content of the biomass sample using Equation (15).

$$Metabolite\ content\ (corrected\ value) = \frac{Metabolite\ content\ (raw\ value)}{1 - Ash\ content - Moisture\ content} \tag{15}$$

2.6.9 Urea concentration

The concentration of urea in the culture medium was measured by using an enzymatic kit (Urea enzymatic, Química Analítica Aplicada S.A., Spain) based on the Berthelot –Searcy method (Foster and Hochholzer, 1971; Wilcox et al., 1966).

2.7 Biological determinations

2.7.1 Maximal photosynthetic efficiency of Photosystem II

Viability of microalgal cultures was measured as the maximal photosynthetic efficiency of Photosystem II (F_v/F_m) and determined according to the method described by Schreiber (Schreiber et al., 1995) using a portable pulse amplitude modulated fluorimeter which measures chlorophyll fluorescence (AquaPen-C AP-C 100, Photon Systems Instruments, Germany) in aliquots of microalgal cultures. F_v represents the minimum level of fluorescence observed in 15 min dark-acclimated cells after the exposure to a non-actinic beam, and F_m is the maximum fluorescence observed in cells following the exposition to a short but strong actinic light pulse.

2.8 Statistics

Unless otherwise indicated, the results show the average values and their corresponding standard deviations of the experimental measurements performed in triplicate. Besides, and unless otherwise indicated, the cultures were performed in duplicate.

For the parameters calculated by the addition, subtraction, multiplication or division of two or more measured values, the standard deviation (SD) of the resulting value was calculated using the error propagation formulas presented below (Equations 16. 17, 18):

For addition and subtraction $z=x+y+\ldots$ or $z=x-y-\ldots$

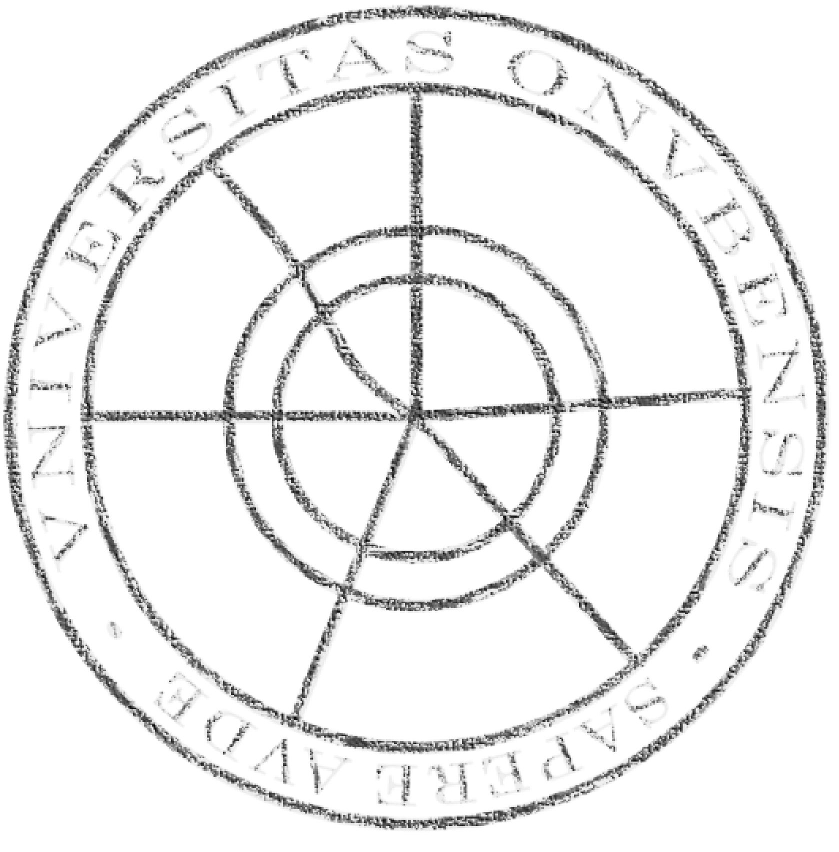
$$SD_z = \sqrt{SD_x^2 + SD_y^2 + \cdots} \qquad (16)$$

For multiplication by an exact number $z = c \cdot x$

$$SD_z = c \cdot SD_x \qquad (17)$$

For multiplications and divisions $z=x \cdot y$ or $z=x/y$

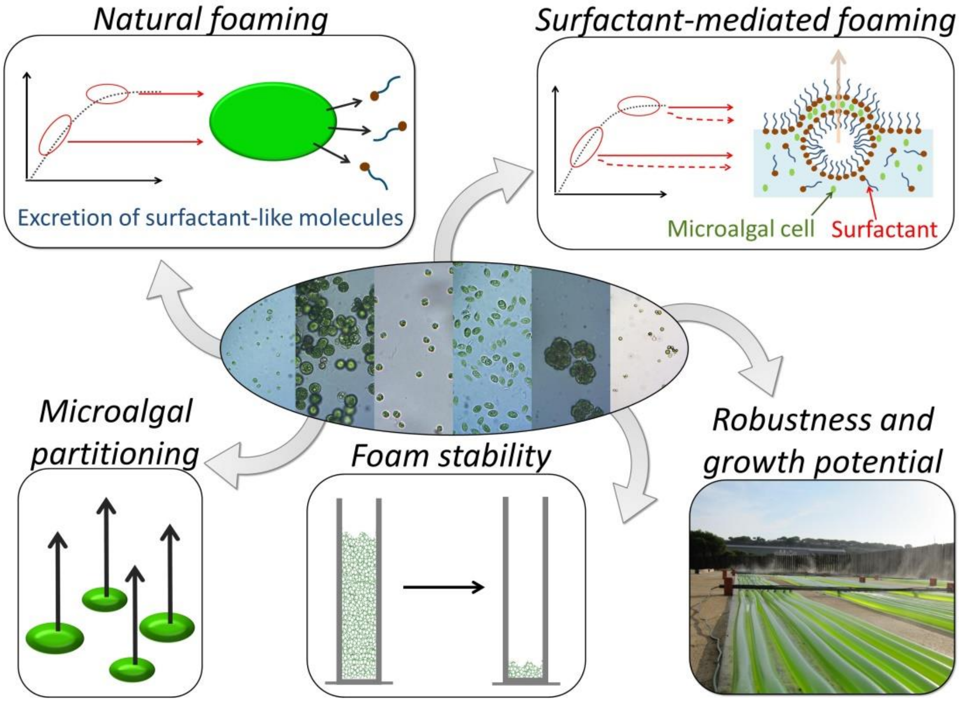
$$\frac{SD_z}{z} = \sqrt{\frac{SD_x{}^2}{x} + \frac{SD_y{}^2}{y} + \cdots} \qquad (18)$$



III. RESULTS AND DISCUSSION

Chapter 3

Selection of microalgae with potential to be cultivated in surfactant-stabilized foams



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3.1 Abstract

Foamability, microalgal partitioning towards the foam and foam stability, together with the inherent characteristics of growth, were established as criteria to assess the potential of a microalgal strain to be cultivated in foam.

The foaming properties mentioned above were studied for 6 algal strains which differ in their nature as well as in their potential uses. Since natural foam production was proved to be unfeasible for all the strains considered, the natural surfactant BSA (bovine serum albumin) was employed to investigate the foaming properties of the microalgal candidates. Culture age, algal hydrophobicity and biomass concentration were shown to influence these foaming properties differently depending on the microalgal strain.

This study revealed large differences in the potentiality of the microalgae to be cultivated in a foam-bed photobioreactor. In particular, among the microalgal strains tested, the commercial strains *Chlorella sorokiniana*, *Nannochloropsis gaditana* and *Scenedesmus obliquus* showed the highest potentiality for cultivation in foam.

Overall, the following criteria could be broadly applied to select suitable microalgae for cultivation in a foam-bed photobioreactor: high or moderate foamability of the microalga-surfactant suspension, microalgal partitioning, stability of the foam formed, and robustness and fast growth of the strains.

3.2 Introduction

The concept of microalgal cultivation in surfactant-stabilized foams has been recently reported as a promising alternative to conventional microalgal cultivation systems (Janoska et al., 2017). It presents several theoretical advantages such as reduction of costs related to harvesting and gas supply, and decreased water consumption, which might overcome some of the main current limitations of microalgal bio-products production.

In a foam-bed photobioreactor, a variety of factors may have an impact on the efficiency of the microalgal biomass cultivation process, which can be classified into three main groups: (i) factors related to the foam system configuration and operational parameters, (ii) characteristics of the microalgal strain used, and, if needed, (iii) the properties of the surfactant employed as wells as its specific interaction with the algal cells.

Concerning the second group, microalgae have the ability to produce natural surfactants that might facilitate their cultivation in foam-bed photobioreactors; however, their production and effectiveness vary considerably among species (Žutić et al., 1981). As examples, the natural foamability of *Chlorella vulgaris* and *Chaetoceros* sp. has been already reported; such capability was used to harvest biomass on a small and large scale via foam flotation (Csordas and Wang 2004; Levin et al., 1962). If natural foam production ability of a cultivated microalga is insufficient, a suitable combination of microalgal strains and surfactants may be required for successful algal cultivation in foam. The usage of surfactants has been so far reported in foam columns or flotation tanks as part of the microalgae harvesting process (Coward et al., 2013; Liu et al., 1999), but only bovine serum albumin (BSA) has been reported to be useful for short-time cultivation of one microalgal species in foam (Janoska et al., 2017). Therefore, it is essential to assess the need for a surfactant addition in algal biomass cultivation for each specific microalgal species.

For a microalgal cell to migrate from the liquid suspension to the foam phase, it has to collide with a bubble and attach to it. However, the collision probability strongly depends on the particle size; and the algal hydrophobicity is a crucial factor for the attachment of the cells to the bubbles (Alhattab and Brooks, 2017; Garg et al., 2012; Hao et al., 2017). In general terms, surfactants can make microalgal surface more hydrophobic, favoring the attachment of the cells to the bubbles, thus determining the flotation performance (Alhattab and Brooks, 2017; Chen et al., 1998; Coward et al., 2013; Garg et al., 2014; Hao et al., 2017; Liu et al., 1999).

Another factor affecting the algal flotation efficiency is the culture age. The charge density of algal cell surfaces, which can influence their interactions with the surfactant, varies significantly throughout the growth phases (Henderson et al., 2008; Liu et al., 1999; Zhang et al., 2012). Moreover, biomass composition tremendously changes during the cultivation period. For example, the excretion of algal molecules with foaming properties, such as proteins or exopolysaccharides, may be enhanced during the stationary phase of growth (Henderson et al., 2008; Lavoie and De la Noüe, 1987; Wang et al., 2014). Proteins are the most frequent surface active biomolecules. In addition to proteins, polysaccharides can improve foam stability by increasing the bulk viscosity, which decreases the rate of disproportion and drainage (Schwenzfeier et al., 2013).

Ionic strength is another key parameter that influences foam flotation processes. High salinity prevents bubble coalescence (Laamanen et al., 2016), which possibly leads to superior foam stability. Conversely, several studies on freshwater microalgae harvesting have indicated that the inner salt concentration negatively affects algal flotation performance (Chen et al., 1998; Liu et al., 1999). This suggests that the potential of freshwater microalgal strains for cultivation in foam might differ from that of marine strains.

Cell concentration may also play a role in the culture foaming properties. For traditional photobioreactors, the viscosity increment due to biomass concentration increase is considered insignificant in terms of the fluid mechanics (Adesanya et al., 2012). However, in a foam-bed photobioreactor a higher biomass concentration can be achieved and the excreted surfactant-like molecules could be accumulated further. Hence, the viscosity increase may play a major role in microalgal cultivation in foam. As mentioned above, the higher the viscosity, the higher the foam stability (Schwenzfeier et al., 2013). On the contrary, an increased viscosity lowers the diffusion of surface-active compounds to the air/water interface, affecting the foamability (Piazza et al., 2008). Moreover, algal cells can be considered as particles that possibly function as foam stabilizers or destabilizers according to their characteristics.

Although most of the principles that may affect the foaming properties of algal suspensions are partially understood, the specific characteristics of different algal strains are expected to influence algal-enriched foam formation and its characteristics. Therefore, this study was aimed at defining criteria to assess the suitability of a given microalgal species to be cultivated in foam. Hence, the following parameters were proposed to be considered: microalgal foamability (natural and mediated by a surfactant), algal partitioning, foam stability and microalgal growth characteristics. Several microalgal strains (particularly different and most of them of commercial importance) were used to compare the identified parameters. Moreover, microalgal cell surface hydrophobicity was studied along the different growth phases and its possible correlation to the microalgal partitioning was analyzed. The effect of medium salinity, culture age and biomass concentration on foaming properties was also investigated because they could influence the formation of algal-enriched, stable foam. To the best of our knowledge, this is the first attempt to experimentally establish criteria which could be systematically used to select microalgae for cultivation in foam.

3.3 Results and discussion

To critically assess the potentiality of a given microalga for cultivation in foam, it is imperative to define the criteria related to the influence of several parameters on the foaming properties of the microalga. Hence, the following parameters were analyzed in few microalgal strain suspensions (particularly different and most of them of commercial importance): natural and BSA-mediated foamability, algal partitioning and foam stability. The joint analysis of the aforementioned parameters together with the inherent growth characteristics of the different algal strains is hereby proposed as the criteria to determine the algae potentiality to be cultivated in foam.

The following six strains were used in this study: *C. sorokiniana*, *B. braunii* CCALA-778, *B. braunii* var. Showa, *N. gaditana*, *N. oleoabundans* and *S. obliquus*. **Table 3.1** enlists some of the main characteristics of these six strains. The microalgal strains were chosen such that they include (i) strains from different salinity habitats (either freshwater or saltwater); (ii) strains differing in growth pattern, either as single cells or forming colonies; (iii) strains covering a diverse cell/colony size range, and, (iv) all microalgal candidate genera were reported to have potentiality to release molecules with foamability behaviour, namely polysaccharides, proteins and/or hydrocarbons (Banerjee et al., 2002; Guil-Guerrero et al., 2004; Jung et al., 2015; Watanabe et al., 2006; Wu et al., 2011). In addition, all the studied microalgal strains have various applications in the field of human health, food and feed, or potentiality for energy production (Banerjee et al., 2002; Da Silva et al., 2009b; Spolaore et al., 2006).

Microalgae from diverse aquatic habitats, marine and freshwater, were included in the present study with the aim of using culture media with different ionic strengths. It is known that increases in salinity lead to the production of smaller bubbles, possibly because salts prevent coalescence by inhibiting the merger of the bubbles (Lin et al., 2011), thereby enhancing foam stability (Laamanen et al., 2016). However, high ionic strength may also affect the interactions between surfactants and algal cells by causing a decrease in algal zeta-potential (Liu et al., 1999). These interactions are crucial for the production of algal-enriched foam. Therefore, the foaming response of the marine and freshwater strains is expected to be different.

Likewise, this study included single cell cultures and the colony-forming strains *S. obliquus* and both *B. braunii* strains. Thus, the microalgal strains also covered a wide range of cell size from 1 µm (*N. gaditana*) to 30-500 µm of (*B. braunii* strains). Cell size influences harvesting of microalgae biomass by flotation, because the efficiency of collision and attachment of a particle to air bubbles depends on the cell size. A small particle size increases the likelihood for the particle to be lifted to the surface. However, a reduction in particle size also decreases the probability of the cells colliding with the bubble (Gerardo et al., 2015). For example, the collection efficiency of ash particles in a column flotation by microbubbles increases as a function of particle size up to 52 µm, but decreases above that size due to the effect of gravitational force effect (Lee and Lee, 2002). Therefore, different cell sizes are expected to affect the foaming properties of algal cultures.

Table 3.1. Main characteristics of the microalgal strains investigated in this study.

Microalga	Characteristics	Extracelullar polymeric substances	Potential or commercial use	References
Botryococcus braunii **CCALA-778 (race A)**	Freshwater sp./Brackish water sp./Saline lakes sp.; Slow growth; Amorphous colonies (30-500 µm)	Polysaccharides	Biopolymers	Banerjee et al., 2002; Bayona and Garcés, 2014; Eroglu and Melis, 2010; Wolf, 1983.
Botryococcus braunii **var. Showa (race B)**		Hydrocarbons	Biofuels production (gasoline, kerosene, diesel)	
Chlorella sorokiniana	Freshwater sp. Single cells, Very fast growth, very robust Average cell diameter: ~1.5-2 µm	Proteins, polysaccharides	Selenomethionine, antioxidants, antitumoral compounds and single cell protein source, wastewater treatment, biofuels production	Becker, 2007; Chung et al., 2012; Gojkovic et al., 2013; Matsukawa et al., 2000; Watanabe et al., 2006.
Nannochloropsis gaditana	Marine sp.; Single cells; Fast growth Average cell diameter: ~1µm	Polysaccharides	Animal feed, human food, biofuels production	Ferreira et al., 2009; Guil-Guerrero et al., 2004; Mitra et al., 2015; Zuliani et al., 2016.
Neochloris oleoabundans	Freshwater sp./Halophile sp.; Single cells whose size are 2-7 µm Moderate growth	Biopolymers	Biofuels production	Da Silva et al., 2009b; De Winter et al., 2013; Li et al., 2008; Wu et al., 2008.
Scenedesmus obliquus	Freshwater sp.; 4-cells colonies; Fast growth, robust Average cell diameter: ~4-8 µm	Proteins, polysaccharides, biopolymers	Wastewater treatment, biofuels production, human food and animal feed	Becker, 2007; Corradi et al., 1998; Da Silva et al., 2009b; Gupta et al., 2016; Jung et al., 2015; Kurniawati et al., 2014; Lavoie and de la Noüe, 1987 Zuliani et al., 2016.

3.3.1 Microalgal growth

In all microalgae-based production processes, fast growth and robustness are desirable characteristics of the cultivated algal strain. In particular, the employment of a robust strain for cultivation in foam would be favorable assuming its inherent tolerance to a given surfactant and its resistance to the shear stress produced by the burst of the bubbles. Similarly, the cultivation of a fast-growing strain would reduce the costs of the process by increasing the productivity. Therefore, these growth characteristics were adopted as one of the criteria employed and proposed in this study to unravel the potentiality of an algal strain to be cultivated in foam.

The microalgal strains were cultivated in batch as described in Materials and Methods (**Section 2.3.1**) and their maximum specific growth rates were calculated.

Despite the cultivation conditions were not optimal for the cultivation of the strains, the comparison of the specific growth rates achieved under the same cultivation conditions give an indication of which species are the fastest growing ones from those hereby studied. In particular, *C. sorokiniana* showed the highest specific growth rate (2.28 d^{-1}), which is significantly higher than those obtained for the rest of the species. This strain is indeed known by its high growth rate (Cazzaniga et al., 2014; Cuaresma et al., 2009). Whereas intermediate values were obtained for *S. obliquus* (1.00 d^{-1}), *N. gaditana* (0.82 d^{-1}) and *N. oleoabundans* (0.71 d^{-1}), low growth rates were found for both *B. braunii* strains (0.52 and 0.30 d^{-1} for the strains CCALA-778 and var. Showa, respectively). *B. braunii* strains are acknowledged to be slow-growing microalgae (Gouveia et al., 2017).

The robustness of a given species refers to its sensitivity to contamination and/or to changes in physicochemical conditions of the culture. According to our observations and experience growing these species, the fastest growing strains – especially *C. sorokiniana* and *S. obliquus*- are also robust strains (e.g. less susceptible to contamination and/or to changes in the physicochemical conditions). Indeed, the robustness of *Chlorella* and *Scenedesmus* strains is denoted by their ability to grow in wastewaters (Li et al., 2011; Tuantet et al., 2014), to grow outdoors at large scale without suffering from collapse or contamination (Basu et al., 2014; Mata et al, 2009), and by their ability to tolerate wide nutrient concentration ranges and changes in temperature or pH (Ji et al., 2013; Kumar et al., 2014), among others.

Despite the fact that the six strains have potential or current applications, differences in growth and robustness exist among the strains investigated, which suggests different potential for these algae to be cultivated in a foam-bed photobioreactor.

3.3.2 Natural foam production

Natural foamability of the microalgal strains was assessed to determine whether such ability possibly minimizes or negates the need for surfactant addition in microalgal cultivation in foam. The natural foam production by an algal culture depends primarily on the production and excretion of surfactant-like molecules that possess foamability behavior. Because some of the key surfactant-like molecules are produced at late linear growth phase and during the stationary phase, late phases of growth could enhance foaming (Henderson et al., 2008; Lavoie and de la Noüe, 1987; Wang et al., 2014). Accordingly, to understand how culture age influences the foamability mediated by accumulation of such surfactant-like molecules at each growth stage, the samples obtained from both linear and stationary phase of growth were tested for foam production.

Natural foamability was calculated as per the method described in Materials and Methods (**Section 2.4.1**). The pH of the samples was adjusted to 7.0 to avoid its influence in the foamability performance. The foam production speeds achieved as a function of the growth phase of the different microalgal strains studied are presented in **Figure 3.1**. As shown, culture age differently influenced the foamability of the microalgal strains. Although the foam production speed of *C. sorokiniana* samples taken at linear growth phase was slightly higher than that taken at stationary growth phase, there was no effect of the growth phase on the natural foam production of *N. gaditana* or *S. obliquus.* On the other hand, both *B. braunii* strains and *N. oleoabundans* showed a higher foamability in samples taken during the stationary growth phase. And maximal foamability was observed in *N. oleoabundans* samples.

The obtained foamabilities were low if compared to reported data from foam flotation experiments based on the natural foaming capacity of different microalgal suspensions (Csordas and Wang, 2004; Levin et al., 1962). However, despite the aforementioned examples, the generally low foamability of microalgal suspensions commonly results in the application of one or more strategies, such as water pressurization, production of microbubbles or the addition of surfactants in order to overcome it. Other studies reported by Coward et al. (2013) for *Chlorella* sp. with cetyltrimethylammonium bromide (CTAB) and Ecover© (Coward et al., 2013) and Chen et al. (1998) for the harvesting of *Scenedesmus quadricauda* using CTAB, Triton X-100 and sodium dodecyl sulfate (SDS) (Chen et al., 1998) are examples of the required addition of a suitable surfactant; hence they showed greater algal-enriched foam production. However, in this study, the foam was naturally produced and, therefore, lower values were obtained.

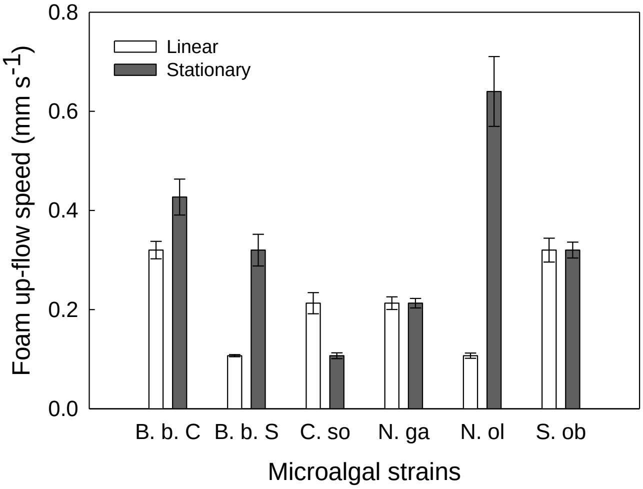


Figure 3.1. Natural foamability expressed as foam up-flow speed (mm s^{-1}) of suspensions of the microalgal strains investigated as a function of the culture age. Microalgal strains: *B. braunii* CCALA-778 (B. b. C), *B. braunii* var. Showa (B. b. S), *C. sorokiniana* (C. so), *N. gaditana* (N. ga), *N. oleoabundans* (N. ol) and *S. obliquus* (S. ob). The samples were taken from cultures in linear growth phase and in stationary phase of growth. The average values of three experimental replicates are shown, together with their corresponding standard deviations.

Overall, despite the differences found among the foam production speeds for the studied microalgal strains, the natural foam produced was not very large or stable to potentially fill a foam-bed photobioreactor of foam. Therefore, to properly study the foamability of the microalgal strains, the addition of a surfactant was deemed necessary; and it was considered a pre-requisite for the potential cultivation of these algal strains in foam.

3.3.3 BSA-mediated foamability

To further compare the foaming properties of different algal suspensions, BSA was used as surfactant because it has good foamability (Clarkson et al., 1999b) and no toxic BSA effects on microalgae have been reported. Moreover, *C. sorokiniana* was proved to grow in BSA-stabilized foams for at least 8 hours (Janoska et al., 2017); therefore, BSA was used to study algal-enriched surfactant-stabilized foams.

Since different marine and freshwater microalgal strains were investigated, the culture media used for their growth had varying NaCl concentrations. However, it was unknown whether the salinity of culture medium exerts any effect on the BSA-mediated foamability of microalgal samples. Therefore, to determine the optimal BSA concentration for these tests, varying BSA concentrations up to 5 g L^{-1} were used for assessing the foam production speeds for the saline (F2 medium) and freshwater culture media (BG11, Chu, and M-8 media).

At BSA concentrations of <0.1 g L^{-1}, the fastest foam production was observed for F2 saline culture medium (**Fig. 3.2**), indicating a positive effect of salinity on foamability when BSA was used as surfactant. Protein foamability can be possibly enhanced at increased NaCl concentration due to the dissociation of protein micelles, the enhancement of protein solubility or lower repulsion between proteins (Zhang et al., 2004). This positive effect of salinity on the foaming capacity has been previously observed for other proteins such as in sesame protein concentrates (Inyang and Iduh, 1996). Moreover, at BSA concentrations of <0.1 g L^{-1}, there were differences in the foaming production speeds among the freshwater culture media (BG11, Chu, and M–8); the M–8 medium had the lowest foam production speed. Therefore, before conducting foaming experiments with algal suspensions, the effect of salinity on foamability must be assessed for each specific surfactant-added culture medium. Furthermore, when BSA concentrations up to 5 g L^{-1} were used, the foam production speeds were similar to those obtained using 0.1 g L^{-1} BSA, indicating that a 50-fold increase in BSA concentration does not increase foam production speed in our system for any of the culture media used. Therefore, to achieve the maximum foam production speed (3.7 ± 0.1 mm s^{-1}) in both freshwater and saline algal culture media, the minimum BSA concentration was determined to be 0.1 g L^{-1}. Hence, 0.1 g L^{-1} BSA was used in this study to compare the foamability of the six microalgal strains in their corresponding culture media.

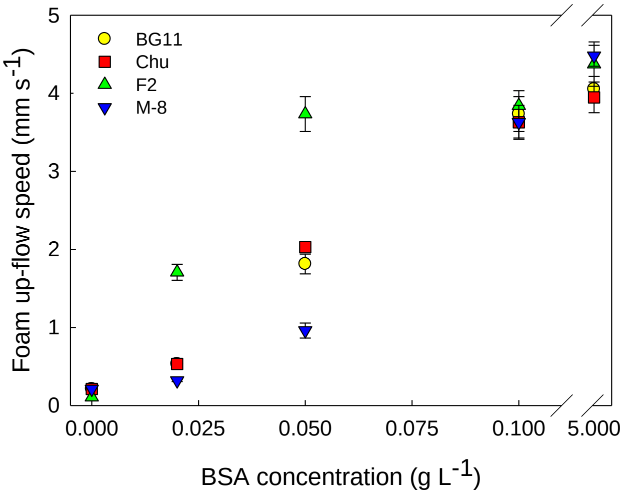


Figure 3.2. BSA-mediated foamability expressed as foam up-flow speed (mm s^{-1}) of the different culture media employed without microalgal cells as a function of BSA concentration. BSA concentrations: 0, 0.02, 0.05, 0.1 g L^{-1}. Freshwater culture media: BG11, Chu and M-8 media. Saline culture media: F2 medium. The average values of three experimental replicates are shown, together with their corresponding standard deviations.

Subsequently, the BSA-mediated foamabilities were determined for each microalga as a function of biomass concentration and the growth phase at which the samples were collected (**Fig. 3.3**). All the foam production speeds for algal suspensions were lower than the maximum speed obtained for their corresponding culture media without cells (3.7 ± 0.1 mm s^{-1}), indicating that the presence of microalgal cells in the culture medium had a negative effect on foam production.

To evaluate the foamability of 3-fold concentrated samples, the algal suspensions were pre-concentrated by centrifugation as described in Materials and Methods (**Section 2.4.1**). In most samples, centrifugation of the culture broth resulted in a null or negative effect on foam production. Although microalgal suspensions were highly diluted, it has been reported that an increase in microalgal cell concentration results in a viscosity increase (Adesanya et al., 2012), possibly providing an explanation for the observed low foamability; viscosity is negatively correlated to foamability (Piazza et al., 2008). Furthermore, some of the surfactant-like molecules released by the algal cells might have been lost in the centrifugation step, thus possibly resulting in lower foamability.

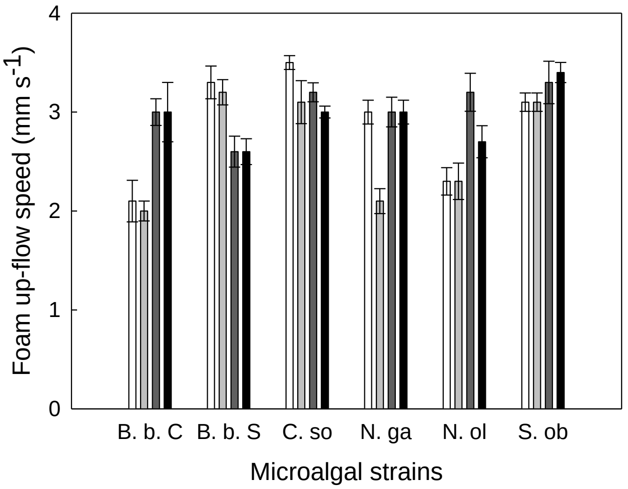


Figure 3.3. BSA-mediated foamability, expressed as foam up-flow speed (mm s^{-1}), of suspensions of the microalgal strains investigated as a function of the culture age and biomass concentration. Microalgal strains: *B. braunii* CCALA-778 (B. b. C), *B. braunii* var. Showa (B. b. S), *C. sorokiniana* (C. so), *N. gaditana* (N. ga), *N. oleoabundans* (N. ol) and *S. obliquus* (S. ob). Experimental conditions: samples from linear growth phase (white bars), samples from linear phase and 3-fold concentrated in biomass (light grey bars), samples from stationary phase (dark grey bars) and samples from stationary phase and 3-fold concentrated in biomass (black bars). The average values of three experimental replicates are shown, together with their corresponding standard deviations.

B. braunii CCALA-778 and *N. oleoabundans* showed much lower foam production speeds when the algal suspensions were collected from cultures in linear growth phase, which is in good agreement with the results of natural foamability discussed above (**Fig. 3.1**). However, *B. braunii* var. Showa, *C. sorokiniana* and *S. obliquus* collected from linear growth phase showed maximal foam production speeds, similar to those obtained for fresh culture media. Minimal or no effects were observed on the foam production speeds for *N. gaditana* and *S. obliquus* cultures collected from the foam phase, similar to the null effect showed on their natural foam production. On the other hand, *B. braunii* var. Showa and *C. sorokiniana* cultures collected from stationary phase showed lower foam production. The lower foam production speed shown by *B. braunii* var. Showa may be attributed to the higher hydrocarbon concentrations in the later phase of growth, which may have had a negative impact on the stability of BSA-stabilized foams. In general, some hydrocarbons possess antifoaming activity and decrease foam production speeds (Lee and Kim, 2004).

Of the microalgal strains tested in this study for a rapid BSA-mediated foam production, *B. braunii* var. Showa, *C. sorokiniana*, and *S. obliquus* were the most suitable microalgal strains according to the BSA-mediated foamability of culture samples taken in the linear growth phase. However, no common pattern was found in the tested microalgae that linked their foaming capacity with any particular characteristic of their algal nature (**Table 3.1**) that emphasizes the need to particularly assess the foamability of each microalgal strain.

3.3.4 Microalgal partitioning and hydrophobicity

One of the main critical requirements for microalgal cultivation in a foam-bed photobioreactor is algal partitioning; this is the ability of the cells to pass from the liquid suspension to the foam phase. Algal partitioning may depend on various factors such as algal cell size, cell surface hydrophobicity, cell concentration, culture age and presence of surfactant-like molecules produced by the algal cells, which are key factors in microalgae flotation studies for algal harvesting (Csordas and Wang, 2004; Gerardo et al., 2015).

To study the effect of biomass concentration, algal partitioning was tested at two biomass concentrations (1.5-2 g L^{-1} and 8-10 g L^{-1}) and samples were taken from linear growth phase cultures. The pH of all the samples was adjusted to 7.0 because it is a major influential parameter affecting the flotation efficiency of microalgal harvesting processes (Csordas and Wang, 2004; Levin et al., 1962).

Figure 3.4 shows large differences in the algal partitioning values obtained for the tested strains. The foam produced by *N. oleoabundans* was not stable enough to perform the algal partitioning assays; therefore it was not used for further studies. Although *B. braunii* var. Showa showed the second highest BSA-mediated foam production speed among all the linear growth phase samples (**Fig. 3.3**), its partitioning was almost null. At a low biomass concentration, the partitioning values for *B. braunii* CCLA-778, *S. obliquus*, *N. gaditana* and *C. sorokiniana* were 0.38, 0.43, 0.60 and 0.89, respectively. At a higher biomass concentration, increased microalgal partitioning was observed; the maximum partitioning value was observed for *C. sorokiniana*, which increased up to 1.52.

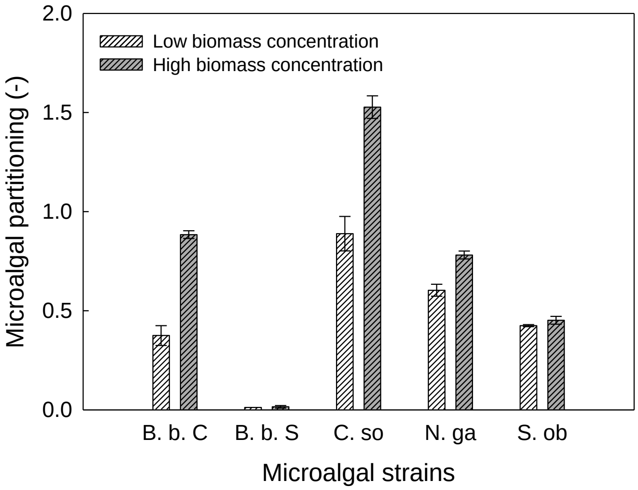


Figure 3.4. BSA-mediated microalgal partitioning (non-dimensional) of the different samples taken from microalgal cultures in linear growth phase as a function of biomass concentration. Microalgal strains: *B. braunii* CCALA-778 (B. b. C), *B. braunii* var. Showa (B. b. S), *C. sorokiniana* (C. so), *N. gaditana* (N. ga) and *S. obliquus* (S. ob). Samples were tested at a low biomass concentration (1.5-2 g L^{-1}) and at a high concentration (8-10 g L^{-1}). The average values of three experimental replicates are shown, together with their corresponding standard deviations.

Among the parameters that influence the partitioning of a surfactant-added algal suspension, hydrophobicity and cell size are inherent to each algal strain. Cell surface hydrophobicity may change depending on the algal growth phase (Henderson et al., 2008), which in turns alter the strength of the interactions between the algal cell surface and the surfactant. Cell surface hydrophobicity is presented in **Figure 3.5** as a function of growth phase. Large differences were found among the hydrophobicity values of the different strains. *S. obliquus, B. braunii* var*.* Showa and *C. sorokiniana* showed the highest hydrophobicity values in the decreasing order. The hydrophobicity of *B. braunii* CCALA-778*, N. gaditana* and *N. oleoabundans* did not exceed 30% in any of the growth phases, making them theoretically less suitable for their cultivation in foam. According to the results, cell hydrophobicity varies to a great extent within strains, oscillating from 0 to almost 100%, which is in agreement with previous findings (Garg et al., 2012).

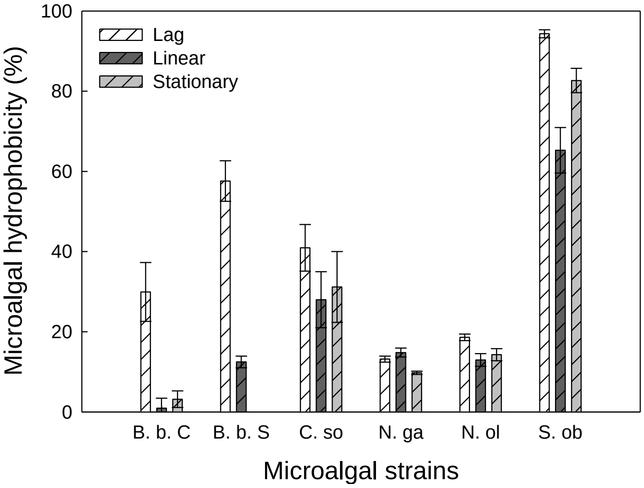


Figure 3.5. Cell hydrophobicity, expressed as percentage, of the microalgal strains investigated as a function of the culture age. Microalgal strains: *B. braunii* CCALA-778 (B. b. C), *B. braunii* var. Showa (B. b. S), *C. sorokiniana* (C. so), *N. gaditana* (N. ga), *N. oleoabundans* (N. ol) and *S. obliquus* (S. ob). Microalgal hydrophobicity was analyzed in samples taken from cultures in lag, linear and stationary growth phase. The average values of three experimental replicates are shown, together with their corresponding standard deviations.

Moreover, slight algal hydrophobicity variations were observed through the different growth phases. The hydrophobicity was minimal during linear growth phase, which is in accordance to the results reported by Zhang et al. (2012) who demonstrated that the density of functional groups, which confers charge density to the algal cell surface, decreases from exponential to stationary growth phases in *C. zofingiensis* (Zhang et al., 2012). However, in this study, despite the oscillations along the growth curves, algal cell surface hydrophobicity was more dependent on the algal strain than on the culture age.

Microalgal partitioning and cell hydrophobicity values with and without the addition of BSA for all the strains studied in linear growth phase are presented in **Figure 3.6**. Particle surface hydrophobicity is an important characteristic required to adhere to the rising bubbles. In general, surfactants are used to modify algal cell surfaces to make them more hydrophobic (Garg et al., 2014). Since the partitioning tests were performed on foam produced from BSA-added samples, the effect of BSA on microalgal hydrophobicities was also studied. Although BSA increased the low natural hydrophobicity of both *B. braunii* strains, it had nearly negligible effects on *C. sorokiniana, N. gaditana* and *S. obliquus.* This indicates that the effects of a particular surfactant on the properties of a specific algal suspension cannot be extrapolated to other microalgal strains.

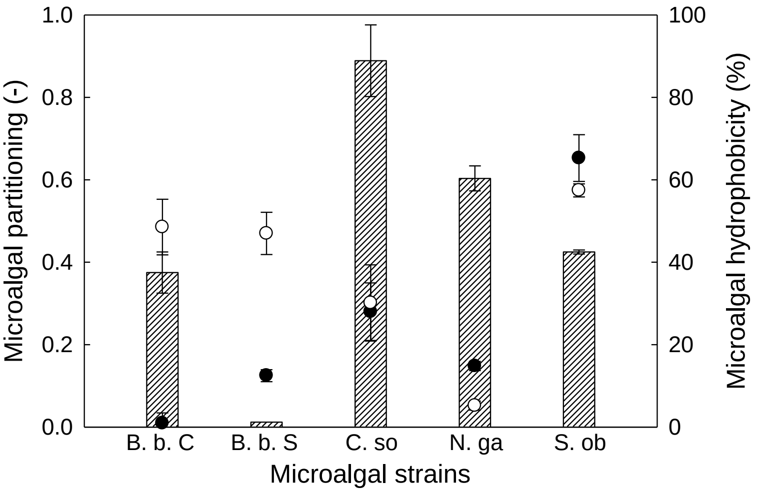


Figure 3.6. Correlation between BSA-mediated microalgal partitioning (vertical bars, non-dimensional) and microalgal cell hydrophobicity (dots, %). Microalgal strains: *B. braunii* CCALA-778 (B. b. C), *B. braunii* var. Showa (B. b. S), *C. sorokiniana* (C. so), *N. gaditana* (N. ga) and *S. obliquus* (S. ob). Black dots: hydrophobicity of samples obtained from cultures in linear growth phase without addition of BSA, and White dots: hydrophobicity of samples obtained from cultures in linear growth phase with the addition of 0.1 g L^{-1} of BSA. The average values of three experimental replicates are shown, together with their corresponding standard deviations.

In this study, neither the natural nor the BSA-mediated algal hydrophobicity showed a strong correlation with algal partitioning for the different microalgal strains investigated. The microalgal hydrophobicities and partitioning values were largely different and contrary for both strains of *B. braunii*, indicating that these two parameters are not necessarily correlated to each other and are strain-specific. The nearly null partitioning of *B. braunii* var. Showa cannot be correlated to its high colony size, *B. braunii* CCALA-778 formed colonies that were similar in size but its partitioning was approximately 0.4. The differences in the composition of their complex extracellular matrix (Banerjee et al., 2002) may have resulted in their distinct partitioning values. Similarly, *C. sorokiniana* cells showed the highest microalgal partitioning, but its BSA-mediated hydrophobicity was below 40%. Although there is a strong correlation between algal hydrophobicity and flotation efficiency (Garg et al., 2012), our results suggest that other factors influence the partitioning of microalgal cells further. The algal hydrophobicity and partitioning values may differ for algal cultivation in foams stabilized by other surfactants, and these should be particularly studied.

3.3.5 Foam stability for the selected microalgal strains

Foam stability was evaluated by comparing the foam lifetime values ($t_{2/3}$, **Table 3.2**) for the different microalgal strains. This parameter indicates the time elapsed for the foam column height to decrease two-thirds of the initial volume. The pH of all the samples was adjusted to 7.0 as foam stability is pH-dependent (Schwenzfeier et al., 2014). In addition, foam collapse speeds were calculated during the first 2 min (**Table 3.2**). Significant differences in foam lifetime and foam collapse speed were found among strains; the most stable foams were produced by *N. gaditana,* followed by *C. sorokiniana, B. braunii* CCALA-778, *B. braunii* var. Showa and *S. obliquus.* As mentioned above, *N. oleoabundans* samples were not used for further assays due to its low foam stability.

Table 3.2. Foam life time ($t_{2/3}$, min) and foam collapse speed (mm s^{-1}) of the microalgal candidates which produce the most stable foams. Microalgal strains: *B. braunii* CCALA-778 (B. b. C), *B. braunii* var. Showa (B. b. S), *C. sorokiniana* (C. so), *N. gaditana* (N. ga) and *S. obliquus* (S. ob). The average values of experimental triplicates are shown, together with their corresponding standard deviations.

	B. b. C	**B. b. S**	**C. so**	**N. ga**	**S. ob**
$t_{2/3}$ (min)	2.52 ± 0.10	1.25 ± 0.10	2.5 ± 0.16	2.94 ± 0.21	1.00 ± 0.06
Foam collapse speed (mm s^{-1})	0.75 ± 0.06	0.93 ± 0.07	0.49 ± 0.03	0.40 ± 0.02	1.12 ± 0.11

Regarding the foam collapse speeds, the colony forming strains (*B. braunii* strains and *S. obliquus*) had a faster foam deconstruction rate, indicating that cell and colony size might have a negative impact on foam stability. In this sense, for a particle to act as effective foam destabilizer, an ideal maximum particle size of 3 µm has been suggested (Kaptay, 2003). Larger particles tend to pierce the foam, promoting foam destabilization (Hunter et al., 2008). Moreover, a correlation between foam destabilization and algal cells hydrophobicity was also found when BSA was used. The BSA-mediated hydrophobicities of the five algal strains which could be compared in terms of their partitioning were in the range of 5-60% (**Fig. 3.6**). The algal strains showing the maximal hydrophobicity values also resulted in the highest foam collapse speeds (both *Botryococcus* and *Scenedesmus* strains).

The hydrophobicity of the particles is crucial for a particle to function as foam stabilizer or destabilizer: the effectiveness of a foam destabilizer increased with the increasing hydrophobicity of the particle (Hunter et al., 2008), which indeed is in good agreement with our findings.

In addition to cell size and cell hydrophobicity, the shape of the particle is an important factor that affects foam stability (Kadoi and Nakae, 2011). Our results indicated that small round single cells of strains such as *N. gaditana* and *C. sorokiniana* led to the lowest foam collapse speeds, which is in agreement with previous statements regarding particles with sharp edges, which enhance its antifoaming action (Dippenaar, 1982), while spherical particles increase foam life time (Kadoi and Nakae, 2011). Smooth particles with low cell surface hydrophobicities are possibly the most suitable for the production of stable algal-enriched foam.

3.3.6. Microalgae selection for algal-enriched surfactant-stabilized foam production

The potential of the microalgal candidates to be cultivated in a foam-bed-photobioreactor was assessed on the analysis of the following parameters that were collectively evaluated: (i) foamability of the alga-surfactant suspension, (ii) microalgal partitioning, and (iii) stability of the foam formed. In addition, growth characteristics inherent to each microalgal strain were also important. The potential of the six microalgal candidates to be cultivated in a foam-bed photobioreactor, according to the above-mentioned foaming properties and their growth characteristics, is summarized in **Table 3.3**. The performance of the different algae for the studied parameters is presented as ″+″ and ″-″ symbols.

A surfactant was required for the cultivation of the six microalgal candidates studied in a foam-bed photobioreactor due to their low natural foamability. Therefore, the BSA-mediated foam production was used in this study as the parameter to compare the foamability of the suspensions for the different microalgal strains. Our results showed that the presence of microalgal cells had a negative impact on foam production compared with cell-free culture media. However, this reduction in foamability varied with the algal strain and the growth phase of the culture (**Fig. 3.3**). For continuous microalgal cultivation in a foam-bed photobioreactor, similarly for cultivation in liquid culture systems, linear growth phase cultures are preferred. Cultures of *B. braunii* var. Showa, *C. sorokiniana*, and *S. obliquus* showed a higher BSA-mediated foamability than the other strains at linear growth phase, indicating that these strains have a larger potential to be cultivated in the foam-bed photobioreactor.

Table 3.3. Comparison of the suitability of the 6 microalgal strains to be cultivated in foam, considering the results of BSA-mediated foamability, microalgal partitioning and foam stability, as well as the algal growth characteristics (robustness, growth rate). Microalgal strains: *B. braunii* CCALA-778 (B. b. C), *B. braunii* var. Showa (B. b. S), *C. sorokiniana* (C. so), *N. gaditana* (N. ga), *N. oleoabundans* (N. ol) and *S. obliquus* (S. ob).

	B. b. C	**B. b. S**	**C. so**	**N. ga**	**N. ol**	**S. ob**
BSA-mediated foam production (linear phase)	+	+++	+++	++	++	+++
Algal partitioning	+	-	+++	++	-	+
Foam stability	+++	+	+++	+++	-	+
Growth characteristics (robustness, fast-growing)	+	+	+++	+++	++	+++

The microalgal partitioning of the algal cells was the second foaming property investigated. In a foam-bed photobioreactor, the foam is produced by bubbling a liquid algal suspension that contains a surfactant and only the foam phase is illuminated (**Chapter 6**). The high algal partitioning allows for the production of foams that are highly enriched with algal cells, which in turn exposes the elevated cell fraction to illumination. In contrast, the low algal partitioning produces foams with poor algal content, which in turn decreases the reactor productivity. Our results showed that for a given surfactant and system, the enrichment in algal cells of the foam formed differs significantly with the algal strain used (**Fig. 3.4**). Although the nearly nonexistent partitioning of *B. braunii* var. Showa dismisses its applicability to be cultivated in foam, *C. sorokiniana* and *N. gaditana* have potential due to their relatively high algal partitioning values. In particular, the highly concentrated samples of *C. sorokiniana* (8.5-10 g L^{-1}), resulted in the maximum algal partitioning value (1.52), indicating that it is a particularly promising strain for cultivation in foam. On the other hand, the moderate partitioning value of *B. braunii* CCALA-778 and *S. obliquus* might be compensated by a good performance of the other criteria evaluated.

After determining the foaming capacity and microalgal partitioning, the stability of foams enriched with the different microalgal strains should also be considered as a criterion for the determination of the potential of a microalga to be cultivated in foam. A high stability of the algal-enriched foam leads to the reduction of the surfactant dosage needed for the cultivation process. Due to the low foam stability, *N. oleoabundans* was excluded from the potential candidates. Small single cells as those of *C. sorokiniana* and *N. gaditana* resulted in the lowest foam collapse speeds; hence, these strains have a larger potential to be cultivated in BSA-stabilized foams as they showed high foam stability.

In addition to the above-mentioned foaming properties, algal growth characteristics possibly play a role in the potential cultivation of a particular strain in foam. Robust strains such as *C. sorokiniana* and *S. obliquus* may be more tolerant to the surfactant as well as more resistant to the shear stress produced by the air bubbles burst. Besides, fast growing strains are preferred to increase the production process productivity. Although the foaming properties of *C. sorokiniana* and *N. gaditana* make these strains clearly more promising than the other algal candidates, *B. braunii* CCALA-778 and *S. obliquus* showed similar performance in terms of their foaming properties. Between these two candidates, *S. obliquus* is a more promising strain that can be cultivated in a foam-bed photobioreactor as it has a faster growth rate and better robustness.

In brief, an algal strain would suit cultivation in foam if exhibits the following criteria: high foamability and foam stability in order to avoid/reduce the addition of a surfactant; high/moderate algal partitioning to enhance biomass transfer from the liquid to the foam phase of the reactor; and high robustness and fast growth to tolerate and favor the cultivation in foam, respectively. According to the abovementioned criteria, *C. sorokiniana*, *N. gaditana* and *S. obliquus* exhibited a higher potentiality for cultivation in foam. Therefore, these microalgal candidates were selected as the most promising strains for cultivation in a foam-bed photobioreactor.

3.4 Conclusions

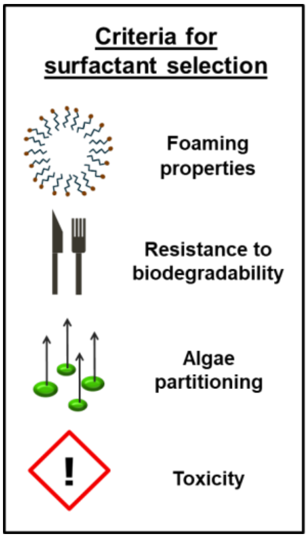
In this study, a major influence of each specific algal characteristic on the production of stable, algal-enriched foam for a given surfactant has been proved and exemplified for several microalgal species with known or potential commercial applications by analyzing of the following parameters: (i) foamability, (ii) microalgal partitioning, and (iii) foam stability. Moreover, the inherent growth characteristics of each microalgal strain were also considered for the final evaluation of the potential of the different strains to be cultivated in a foam bed-photobioreactor.

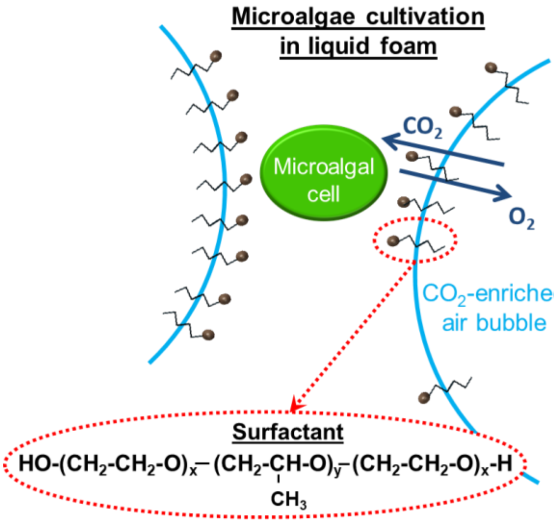
The natural foamability of the six microalgal strains was insufficient for sustaining continuous and stable foam production; hence, surfactant was added. *C. sorokiniana*, *N. gaditana* and *S. obliquus* showed better properties to produce stable, algal-enriched foam in BSA-added cultures.

The specific assessment of the abovementioned parameters for different microalgal strains helped in defining of the following criteria to evaluate the potential of a microalgal strain to be cultivated in algal-enriched stabilized foams: high/moderate foamability, algal partitioning and foam stabilization by the algal suspensions, and high robustness and fast growth of the algal cells. These criteria might have a broad range of applications. To the best of our knowledge, these are the first guidelines that aid in selecting microalgal species suitable for their cultivation in foam bed-photobioreactors. This novel microalgal cultivation concept might boost the microalgal industry further.

Chapter 4

Surfactant selection for microalgae cultivation in surfactant-stabilized foams





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4.1 Abstract

The addition of a surfactant has been deemed necessary to cultivate microalgae in foam. Such surfactant should fit several requirements to be suitable for its employment in a liquid-foam-bed photobioreactor: (i) the surfactant should have good foaming properties, (ii) it should drag-up the microalgae in the foam formed, (iii) it should not be rapidly biodegradable, and (iv) it should not be toxic to microalgae.

The four criteria mentioned above were hereby employed to assess the suitability of ten different surfactants (non-ionic, anionic and cationic), using three microalgal strains with potential to be cultivated in foam: *Chlorella sorokiniana, Nannochloropsis gaditana* and *Scenedesmus obliquus*.

The results obtained in this study indicated that the Pluronics –especially Pluronic F68- were the most promising surfactants according to the criteria assessed. These findings open possibilities for microalgae cultivation in Pluronic-stabilized foams in liquid foam-bed photobioreactors.

4.2 Introduction

The concept of a liquid foam-bed photobioreactor has been recently developed as an innovative and low-cost alternative for microalgae cultivation (Janoska et al., 2017). The natural foam production ability of six microalgal strains has been proved to be insufficient for the performance of a foam-bed photobioreactor, therefore the addition of a surfactant has been deemed necessary (Chapter 3). Before this Thesis, microalgae cultivation in foam had been only achieved in BSA-stabilized foams, although the cultivation time was limited to several hours due to the short-lived foamability of BSA (Janoska et al., 2017). The selection of a suitable surfactant is therefore crucial for successful long-term microalgal cultivation in foam. However, surfactants (i.e. foam stabilizing agents) are a highly diverse group of chemicals that vary in their electrostatic charge (non-ionic, cationic, anionic, zwitterionic), as well as in their nature (biological, synthetic). The chemical diversity of the surfactants results in a wide variety of surface-active compounds with highly differing characteristics, such as foaming properties, biodegradability and their type of interaction with biological surfaces.

In this study, the potential of surfactants to be employed in a foam-bed photobioreactor was assessed according to four characteristics: (i) foaming properties, (ii) microalgal partitioning, (iii) biodegradability, and (iv) toxicity to microalgae.

The foaming properties of the surfactant are important since they determine the concentration at which it should be used for good performance of the foam-bed photobioreactor. The perfect foam should have intermediate liquid content and stability in order to ensure that enough foam can be produced to fill the reactor but the foam is also breakable. Besides, the foam should be homogeneous and it should be composed of small bubbles in order to maximize mass transfer. These properties are dependent on the surfactant structure, e.g. the length of the hydrophobic chain was shown to influence the surface activity and motility of the surfactant, determining the foam properties (Beneventi et al., 2001). Moreover, the surfactant should have the capacity to produce foam repeatedly; therefore, the surfactant molecules must keep their foamability once they are released from the broken foam.

The second characteristic is the partitioning of microalgae towards the foam phase. It is evident that the foams formed by the chosen surfactant must be enriched with microalgae. In general, surfactants are used to render the algal cell surfaces hydrophobic (Chen et al., 1998; Coward et al., 2013; Garg et al., 2014; Liu et al., 1999). The hydrophobic surfactant-coated algal cells can interact with the hydrophobic bubbles via hydrophobic interactions, thus increasing microalgal partitioning (Garg et al., 2013). It has been shown that microalgal partitioning depends on the algal strain (**Fig. 3.4**). Besides, for a given algal strain, the specific interactions between the particular surfactant and the cells are expected to influence the partitioning. In particular, cationic surfactants are presumed to result in higher algal partitioning as they attach the strongest to the usually negatively charged algal cell (Phoochinda and White, 2003).

The third characteristic for surfactant selection is the biodegradation rate of the surfactant. Many surfactants can be biologically degraded (Jurado et al., 2013; Scott and Jones, 2000), which means that microorganisms can either utilize them as substrates for energy and nutrients or they can be co-metabolized by metabolic reactions (Ying, 2006). Biodegradation is directly related to the structure of the molecule (Ivanković and Hrenović, 2010; Jurado et al., 2007). For example, long, aromatic or branched hydrocarbon tails results in difficulties for the surfactant molecules to penetrate into the cells and to be biodegraded (Dorn et al., 1993; Nyberg, 1988). Considering different bacteria populations grow naturally associated to growing microalgae (Fuentes et al., 2016), the optimal surfactant should show a low biodegradability in order to minimize surfactant losses associated to its bacterial consumption.

Finally, for a surfactant to be utilized in a liquid foam-bed photobioreactor, the absence of toxic effects is essential. A variety of toxic effects of surfactants on microalgae have been reported, including reduction in cell density, growth rate, cell motility, chlorophyll content, and the inhibition of the photosynthetic activity (Azizullah et al., 2012, 2014; Garrido-Pérez et al., 2008; Maksimov and Parshikova, 2006). The toxicity of a surfactant is highly specific of the type of surfactant and the organism tested (Ivanković and Hrenović, 2010). For example, cationic surfactants are, in general, more toxic than their anionic or non-ionic partners (Lewis, 1990).

Although some basic principles are understood, it is still difficult to predict surfactants properties and their interactions with different algal species solely based on their structure and theory. The aim of this study was, therefore, to empirically select a suitable foam stabilizing agent for the liquid foam-bed photobioreactor that could enable elongated microalgal cultivations of days to weeks. In this sense, the potential of ten different surfactants was evaluated in terms of measured foaming properties, algal partitioning towards the foam, biodegradability and toxicity to microalgae. For this, *Chlorella sorokiniana*, *Nannochloropsis gaditana* and *Scenedesmus obliquus* were used as microalgal strains according to their large potential to be cultivated in foam (Chapter 3). Based on these results the best surfactant for the application in foam-bed photobioreactors was selected.

4.3 Results and discussion

4.3.1. Foaming properties

Foam volume half-life, liquid hold-up and bubble size were measured for the foams stabilized by the different surfactants at 1 and 5 critical micelle concentrations (CMC) (**Fig. 4.1 A-C**). A concentration of 1 CMC is the minimal concentration required to reach the maximal surface tension reduction and, hence, to attain good foaming characteristics (Boonyasuwat et al., 2005). A concentration higher than 1 CMC (i.e. 5 CMC) was employed to avoid surfactant depletion in the liquid phase during the experimentation.

Figure 4.1A shows that CTAB and Saponin formed the most stable foams at 5 CMC. The exact foam volume half-life values of these surfactants could not be determined since they were longer than two hours, which was the duration of the experiments. Long foam volume half-life was also obtained for BSA and Tergitol NP9 at 5 CMC. At this concentration intermediate foam stability was found for both Pluronics and Triton X-100. Tergitol TMN6 and Tween 20 showed rapid destabilization at 5 CMC, as exemplified by a foam half-life shorter than 10 min. SDS also resulted in unstable foams and at 1 CMC it did not reach the foam height required for the assay. In general, foam stability decreased with decreasing surfactant concentration from 5 to 1 CMC, possibly due to the depletion of surfactants molecules in the liquid phase. Surfactants are amphiphilic molecules that adsorb into the liquid/gas interphase and partition into the foam phase when the liquid is sparged with gas bubbles, thus the surfactant concentration in the liquid phase diminishes while foam is produced. The effect of surfactant depletion is especially relevant when the initial surfactant concentration is low (Boos et al., 2012). Moreover, as the surfactant concentration decreases in the bulk liquid, the rate of diffusion of the surfactant molecules to the interface may also influence foam stability. When the surfactant transport rate is low, foam is formed with reduced amount of adsorbed surfactant molecules, thus the foam stability can also be reduced (Carey and Stubenrauch, 2009). Finally, larger foam stability above CMC might be explained by the presence of micelles in the thin films (Beneventi et al., 2003). Consequently, at higher surfactant concentrations (>CMC) more stable and more wet foam can be formed.

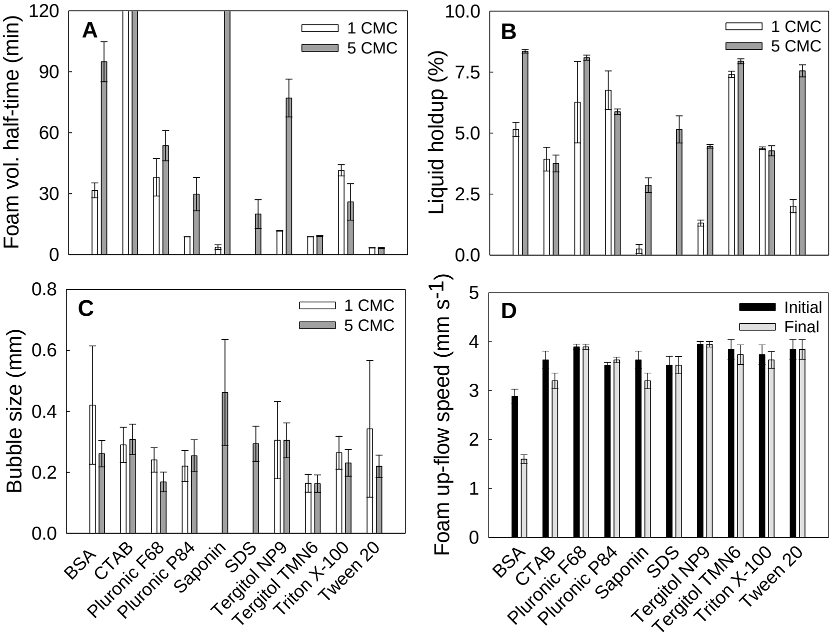


Figure 4.1. Surfactants foaming properties: (A) Foam stability, expressed as foam volume half-life (min), which is the time elapsed when the foam volume has collapsed to its half. (B) Liquid holdup in foams stabilized by different surfactants, expressed as the volume percentage of liquid incorporated in the foam (%). (C) Bubble size of foams formed by different surfactants (mm). (D) Surfactants stability, represented as the initial and final foam up-flow speed of surfactant solutions after 20 cycles of repeated foam formation and deconstruction (mm s^{-1}). Parameters A, B and C were analyzed at two different surfactant concentrations (1 CMC and 5 CMC). SDS and Saponin at 1 CMC did not form sufficiently stable foams for the analysis of bubble size. Liquid holdup and foam stability could not be measured with SDS at 1 CMC due to its low foaming ability. The average values of experimental duplicates are shown, together with their corresponding standard deviations.

Figure 4.1B presents the liquid holdup measured in the foam. BSA, Pluronic F68, Tergitol TMN6 and Tween 20 at 5 CMC produced the foams with the highest liquid holdup, with values between 7.5 and 8.5%. At 5 CMC, the lowest liquid holdup was 2.9% for Saponin foams. In general, lower liquid holdup was obtained at 1CMC in comparison with 5 CMC (**Fig. 4.1B**), which indicates the influence of surfactant concentration on the liquid content of the foam (Lu et al., 2010; Stevenson, 2007). However, no differences in the liquid holdup were observed for CTAB, Pluronic P84 and Triton X-100 at 1 and 5 CMC. In these cases, the surfactant concentrations employed might have been higher than the concentration range where the liquid holdup depends on the surfactant concentration (Lunkenheimer and Malysa, 2003).

Next to foam stability and liquid holdup, the bubble size was analyzed (**Fig. 4.1C**). In general, the bubble size was similar at 1 and 5 CMC. The smallest bubbles (<0.2 mm radius) were found in Tergitol TMN6-stabilized foams. The largest bubbles were formed by Saponin at 5 CMC, with almost 0.5 mm average bubble radius. The standard deviations in **Figure 4.1C** can be correlated with the uniformity of the foam: a high error bar means the foam was heterogeneous, while small deviations indicated that the foam was homogeneous in terms of the bubble size. In heterogeneous foams, the difference of pressure among bubbles with different sizes (small bubbles pressure > large bubbles pressure) causes the diffusion of gas from the smaller bubbles to the larger ones. This phenomenon is known as disproportionation (Pugh, 1996) and generally promotes foam instability (Lemlich, 1978). Overall, smaller bubble sizes were related to increased liquid holdup values (**Fig. 4.1B** and **4.1C**). It can be concluded that increased surfactant concentration results in the combined effects of increased liquid holdup and foam stability, which is in accordance with previous observations (Deshpande and Barigou, 1999).

In addition to foam stability, liquid content and bubble size, surfactant stability against foaming was measured. To this end, the minimum concentration at which the surfactants achieved the maximum foam production speed (C_{min}) was experimentally determined and it is shown in **Table 4.1**. To determine the surfactants stability, twenty repeated cycles of foam production and deconstruction were performed for solutions of each surfactant at 1.1 C_{min}. A concentration higher than C_{min} (i.e. 1.1 C_{min}) was employed since the surfactants might attach to the foam system, therefore producing possible decreases in the foam production speed which would not be due to foamability losses. The results are shown in **Figure 4.2**. Besides, the foam production speeds achieved during the first and the twentieth cycle of foam production and deconstruction are shown in **Figure 4.1D**.

Table 4.1. Surfactant concentrations (CMC) assessed for the determination of C_{min}. The concentration selected as C_{min} is indicated by "*".

Surfactant	Surfactant concentration (CMC)	Foam up-flow speed (mm s^{-1})
BSA	2.00	1.49 ± 0.00
	3.00	1.60 ± 0.00
	4.00	1.76 ± 0.08
	4.75	2.24 ± 0.00
	5.00*	2.88 ± 0.00
	6.00	2.83 ± 0.08
CTAB	0.10	2.99 ± 0.15
	0.25	3.52 ± 0.00
	0.50*	3.63 ± 0.00
	0.75	3.63 ± 0.15
Pluronic F68	2.50	3.36 ± 0.08
	3.75	3.41 ± 0.00
	4.50	3.47 ± 0.08
	5.00*	3.63 ± 0.00
	7.50	3.63 ± 0.00
Pluronic P84	3.00	3.47 ± 0.08
	3.50	3.57 ± 0.08
	4.00	3.68 ± 0.08
	4.25*	3.89 ± 0.06
	4.75	3.89 ± 0.08
	5.00	3.79 ± 0.08
	5.50	3.84 ± 0.00
Saponin	0.50*	3.70 ± 0.06
	1.00	3.66 ± 0.06
	1.50	3.70 ± 0.06
	1.75	3.66 ± 0.00
SDS	0.025	3.20 ± 0.11
	0.050	3.27 ± 0.06
	0.075*	3.48 ± 0.06
	0.150	3.48 ± 0.06

Table 4.1. *(Continuation).*

Surfactant	Surfactant concentration (CMC)	Foam up-flow speed (mm s-1)
Tergitol TMN6	0.25	3.27 ± 0.27
	0.50	3.38 ± 0.27
	0.75	3.95 ± 0.00
	1.00*	4.05 ± 0.11
	1.25	3.91 ± 0.16
Tergitol NP9	0.50	3.57 ± 0.08
	0.75	3.73 ± 0.00
	1.00*	3.84 ± 0.00
	1.25	3.84 ± 0.00
	1.50	3.84 ± 0.00
Triton X-100	0.10	2.56 ± 0.00
	0.25	3.09 ± 0.00
	0.50	3.39 ± 0.10
	0.75*	3.73 ± 0.00
	1.00	3.73 ± 0.00
Tween 20	0.25	2.95 ± 0.06
	0.50*	3.63 ± 0.00
	0.75	3.68 ± 0.08
	1.00	3.73 ± 0.00

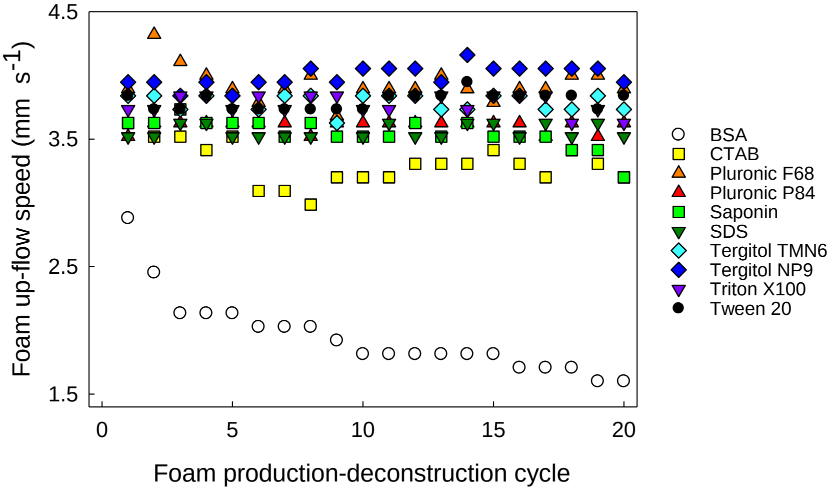


Figure 4.2. Surfactant foamability expressed as foam up-flow speed (mm s^{-1}) measured after 30 s of foaming, along twenty cycles of repeated foam production and deconstruction. The average values of experimental triplicates are shown.

Foam production speed was measured as the foam up-flow speed achieved during the first 30 seconds of foaming. A reduction in this parameter indicates a loss of surfactant foamability, and therefore, of surfactant stability. No significant decrease in the foamability was found for any surfactant except for BSA and CTAB. Previous research found that BSA is not able to produce foams for elongated time in a foam-bed photobioreactor (Janoska et al., 2017) and indicated that BSA could have been denaturized due to foaming or because of degradation by bacteria. Our results showed that the foamability of BSA decreased steeply from the first foaming cycle (**Fig. 4.2**), which denotes the impact of foam production-deconstruction on its foaming capacity. BSA, as any other surfactant, adsorbs at the liquid/gas interphases (Clarkson et al., 2000). Upon surface adsorption, BSA (like other proteins) undergoes conformational changes to expose its hydrophobic residues towards the gas phase (Clarkson et al., 2000; Wilde et al., 2004), which is known as surface denaturation. For BSA, surface denaturation mostly consists in changes in the tertiary structure of the protein (Clark et al., 1998). Our results showed that the foamability of BSA solution decreased by half after twenty cycles of foam production and deconstruction. It has been reported that 10% of the BSA molecules that are adsorbed and desorbed suffer denaturation (Clarkson et al., 1999a), which indicates that some BSA molecules remained in the bulk liquid in each cycle of foam production and deconstruction. On the other hand, the foamability of CTAB fluctuated along the 20 cycles (**Fig. 4.2**) of repeated foam production and deconstruction, resulting in a slightly lower foamability at the end of the experiment (**Fig. 4.1D**).

4.3.2. Microalgal partitioning

Microalgae partitioning towards the foam phase was measured for *Chorella sorokiniana* and *Scenedesmus obliquus* in foams stabilized by the different surfactants (**Fig. 4.3**). The partitioning is defined as the ratio between the algae concentration in the foam and the algae concentration of the suspension before foaming. A partitioning value of one (1.0) indicates that the algal concentration in the foam phase is identical to that in the liquid prior foam formation, implying that it is also identical to that in the underlying bulk liquid as can be calculated from a microalgae mass balance over the system.

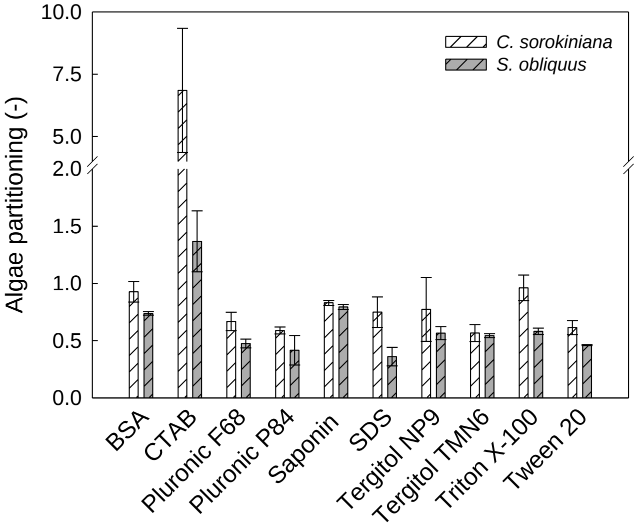


Figure 4.3. *Chlorella sorokiniana* and *Scenedesmus obliquus* partitioning towards the foam phase. The experiments were carried out at the surfactant concentration of 5 CMC (except for Saponin, which at 5 CMC did not generate enough foam for the measurements; thus, 10 CMC was used). BSA was tested at a concentration of 1.75 g L^{-1}. The microalgae concentration was adapted to 15-20, expressed in O.D. at 750 nm, for *C. sorokiniana* and 2 for *S. obliquus*. The average values of experimental duplicates are shown, together with their corresponding standard deviations.

Partitioning values higher than one (1.0) were only obtained in foams stabilized by the cationic surfactant CTAB. In these foams, the microalgal concentration was 6.8 and 1.4-fold higher than in the liquid phase for *C. sorokiniana* and *S. obliquus*, respectively. In general, the microalgal cells are negatively charged (Kim et al., 2017; Rao et al., 2018). Thus, stronger interactions might take place between the cells and the surfactant if the latter is positively charged, hence, the subsequent electrostatic interactions between the cell-surfactant aggregates and the air bubbles might possibly be improved when using a cationic surfactant (Alhattab and Brooks, 2017; Coward et al., 2014). Partitioning values below one (1.0) were achieved with all the other surfactants. This is in accordance to previous observations of better flotation performances of *Chlorella* and *Scenedesmus* strains obtained with CTAB compared to anionic and non-ionic surfactants (Chen et al., 1998; Liu et al., 1999; Phoochinda and White, 2003). The other surfactants (anionic, non-ionic and natural surfactants) led to a low partitioning of both microalgae towards the foam. In SDS-stabilized foams, electrostatic repulsion forces are expected between the algal cell surface and the anionic surfactant

(Phoochinda et al., 2004), and these repulsion forces are probably behind the lowest partitioning value found for *S. obliquus.*

Hydrophobic interactions might occur between the non-ionic surfactants and the algal cells (Chang et al., 2005; Rosen and Li, 2001). As example, they are able to interact with the apolar aminoacids via hydrophobic interactions and with the polar residues of proteins via hydrogen bonding or electrostatic interactions (Cserháti, 1995). These interactions are expected to be surfactant-alga specific and might result in the different partitioning values observed.

In general, *C. sorokiniana* achieved higher partitioning values than *S. obliquus* (**Fig. 4.3**), which is in accordance with our previous results obtained in a different experimental system (Chapter 3, **Fig. 3.4**; Vázquez et al., 2018). Cell surface hydrophobicity has been stated as an important factor affecting microalgal flotation (Garg et al., 2015). However, it was previously proved that the partitioning values of these strains in BSA-stabilized foams are not correlated with their natural or their BSA-added cell surface hydrophobicities (**Fig. 3.6**; Vázquez et al., 2018). Hydrophobicity values of 30 and 65% were found for *C. sorokiniana* and *S. obliquus* respectively (**Fig. 3.6**), therefore, higher partitioning values might be expected for *S. obliquus.* Thus, although the hydrophobicity of these strains was not measured in the presence of the other surfactants, other parameters such as cell size or shape might be responsible for the better partitioning performance of *C. sorokiniana*.

4.3.3. Surfactants biodegradability

Surfactants susceptibility to bacterial degradation (i.e. biodegradation) was indirectly determined by following bacterial growth in the absence of microalgae (**Fig. 4.4**). Surfactants solutions were inoculated with the supernatant of a *Scenedesmus obliquus* culture, which naturally contains microalgae-associated bacteria. The bacteria associated to microalgal cultures are strain-specific (Fuentes et al., 2016), thus bacteria isolated from different microalgal cultures might result in different biodegradation behavior. In this study, the bacteria were isolated from *S. obliquus* cultures because it showed to be the most tolerant strain to the surfactants evaluated (**Section 4.3.4**); therefore, its potential for microalgal cultivation in foam was considered the highest at the time of these experiments. Algal cells were removed from the inoculum by centrifugation, since organic molecules linked to the algal cells and decaying algal biomass might result in additional carbon source for bacterial growth. The fresh water algal culture medium M-8 was enriched with each surfactant at concentrations of 1 and 10 CMC, since concentrations within these limits are expected to be applied in a foam-bed photobioreactor. The concentration of 1 CMC still allows for acceptable foam formation and possible growth inhibitory effects on bacterial cells are minimized. A high concentration of 10 CMC was chosen in order to assess promoting and/or inhibiting surfactant effects on bacterial growth. As previously commented, the surfactants added were the only reduced carbon source available to promote bacterial growth. Thus, bacterial growth, if observed, should be a direct evidence for bacterial degradation of the surfactants as source of carbon and energy.

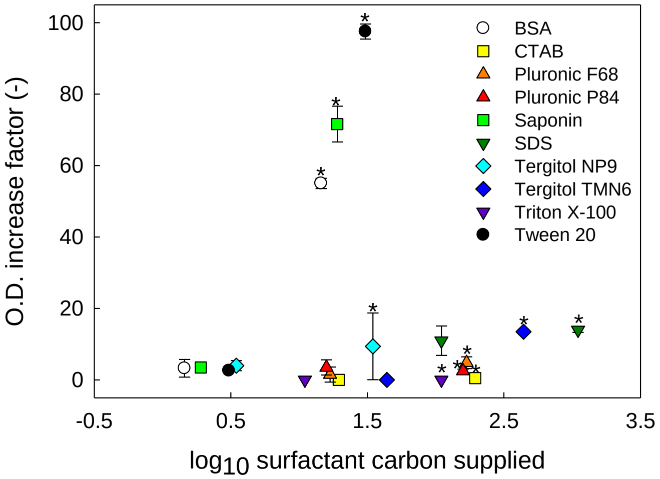


Figure 4.4. Surfactants biodegradability, represented as the bacterial growth on surfactant solutions after three days of cultivation at 1 and 10 CMC surfactant concentrations. The "*" indicates data obtained at 10 CMC. Bacterial growth is expressed as the increase in optical density (600 nm) after three days and it is plotted against the carbon dosage resulting from surfactant addition (logarithmic values, in milligrams). Optical density values were corrected with their corresponding blanks (culture medium with surfactant) and optical density increments were normalized with respect to their initial optical densities. The average values of duplicate cultures are shown, together with their corresponding standard deviations.

Figure 4.4 shows results of bacterial growth on the different surfactants after three days of cultivation at two different concentrations (1 and 10 CMC). The surfactants evaluated have varied CMC values and chemical compositions (**Table 4.2**). Thus, the carbon supplied by each surfactant differed when added at the same concentration in CMC units, as shown in **Table 4.2.** Subsequently, in order to strictly compare the surfactants in terms of the carbon content supplied, the X-axis of **Figure 4.4** is expressed as the decimal logarithm of the milligrams of carbon added to each culture, calculated from the carbon content of the surfactants.

According to **Figure 4.4**, the lowest bacterial growth was obtained in the culture medium containing either CTAB, both Pluronics, or Triton X-100. For these surfactants no differences were observed at the two concentrations tested. Despite their high carbon content, compared to other evaluated surfactants, the bacterial growth was minimal and, therefore, they can be considered less susceptible to biodegradation. The cultures that did not show any growth remained unchanged even after two weeks (data not shown).

A surfactant can be not biodegradable due to the inability of the microorganisms to metabolize it or because it is toxic. In the latter case, microorganisms are also inhibited from using any other degradable carbon source (Zeng et al., 2007). From this assay it was not possible to distinguish if these surfactants were hardly biodegradable or toxic to the bacteria, since the surfactants were the only carbon source present in the cultures. A study reported by Zeng and co-workers (2007) showed that CTAB and Triton X-100 could not be biodegraded by representative Gram-positive (*Bacillus subtilis*), Gram-negative (*Pseudomonas aeruginosa*) bacteria or waste compost bacteria. They showed that whereas CTAB inhibited the growth of all the tested microorganisms, Triton X-100 allowed all of the microorganisms to grow using glucose as an additional carbon source, thus Triton X-100 was considered hardly biodegradable. Besides, Pluronics have been reported to be biorecalcitrant (Adams et al., 1996), which refers to the inability of biodegradation of these compounds (Marsolek et al., 2007), and therefore their inability to sustain bacterial growth.

Table 4.2. Critical Micelle Concentration (CMC, g L^{-1}), molecular weight (g mol^{-1}), molar ratio of the elements that comprise the surfactants, carbon content (% w/w) and carbon supplied by the different surfactants at 1 and 10 CMC concentration (mg L^{-1}).

Surfactant	CMC (g L^{-1})	Molecular weight (g mol^{-1})	Chemical composition (molar ratio)							Carbon content (% w/w)	Carbon supplied (mg L^{-1})	
			Br	C	H	N	Na	O	S		1 CMC	10 CMC
BSA	0.030	2754.11		123	193	35		37		53.64	0.016	0.161
CTAB	0.346	364.45	1	19	42	1				62.62	0.217	2.167
Pluronic F68	0.334	9000.00		420.4	840.7			194.7		56.10	0.187	1.874
Pluronic P84	0.298	4000.00		196.9	393.7			77.7		59.11	0.176	1.763
Saponin	0.050	486.69		30	46			5		42.73	0.021	0.214
SDS	2.451	288.37		12	25		1	4	1	49.98	1.225	12.25
Tergitol NP9	0.060	616.82		33	60			10		64.26	0.039	0.386
Tergitol TMN6	0.800	318.50		18	38			4		61.09	0.489	4.887
Triton X-100	0.194	646.85		34	62			11		63.13	0.122	1.225
Tween 20	0.060	1228.00		58	114			26		56.73	0.034	0.340

On the other hand, the bacterial growth was by far more intense in the cultures containing BSA, Saponin and Tween 20 at 10 CMC, suggesting that indeed the carbon contained in these surfactants was used as substrate for bacterial growth. The ability of bacteria to degrade these surfactants has been already reported (Chang et al., 2014; Confer and Logan, 1997; Mølgaard et al., 2000). In general, molecules as large as BSA (65 kDa) cannot be transported across the bacterial cell wall due to its high molecular weight (Cadoret et al., 2002). The mechanism that has been proposed for the biodegradation of macromolecules such as BSA consists of its hydrolysis on the bacterial surface by extracellular enzymes, followed by the release of the smaller units. These steps are repeated until the hydrolytic fragments are small enough to be directly assimilated by the cells (Confer and Logan, 1997; Mosquera-Corral et al., 2003). Peptides smaller than 1 kDa can be transported across the bacterial cell wall via specific transporters (Cadoret et al., 2002; Wang et al., 2018), such as proton coupled peptide transporters and ATP-binding cassette (ABC) peptide transporters (Garai et al., 2017). The biodegradability of Saponin was expected since biosurfactants are considered environmentally friendly and biodegradable (Soberón-Chávez and Maier, 2011). In this sense, the biodegradable character of Saponins is well acknowledged (Schmitt et al., 2014), and complete biodegradation of this biosurfactant has been already reported (Mølgaard et al., 2000). Tween 20 has also been reported as biodegradable by bacteria in both aerobic and anaerobic conditions (Chang et al., 2014; Yeh et al., 1998).

A third group of surfactants which showed an intermediate biodegradability could be inferred from **Figure 4.4**. In this sense, both Tergitols and SDS promoted moderate bacterial growth. Bacterial growth was independent of the initial carbon content when SDS was used, which might indicate low efficiency of bacteria to degrade such surfactant and/or a possible toxic effect. Finally, regarding both Tergitols, a higher concentration of TMN6 was needed to promote the same bacterial growth as NP9, which might indicate that Tergitol NP9 is slightly more readily biodegradable. The mechanism of biodegradation of APE (alkyl phenol ethoxylates) surfactants, such as Tergitol NP9, is known and it consists in the shortening of the ethoxylated (EO) chain, followed by oxidation of the resulting short chain, yielding more persistent shorter-chain APEs and alkylphenols (APs), which are resilient to degradation by microorganisms (Chokwe et al., 2017;

Ivanković and Hrenović, 2010; Scott and Jones, 2000). Thus, a partial biodegradation of Tergitol NP9 might explain the moderate bacterial growth observed in this case. On the other hand, Tergitol TMN6 has a very branched structure (**Fig. 2.1**) which might be the reason behind their low biodegradation; biodegradability is negatively correlated to the extent of branching (Dorn et al., 1993; Jurado et al., 2007).

The bacterial ability to degrade a surfactant and use it for growth depends on the chemical structure of the surfactant (Scott and Jones, 2000). Three of the evaluated surfactants have fatty-alcohol ethoxylate structure (FAE; commercial names: Pluronic F68, Pluronic P84, Tergitol TMN6), and two of them have alkylphenolpolyethoxylate structure (APE; commercial names: Tergitol NP9 and Triton X-100). Under aerobic conditions, most surfactants can be degradable, including FAE and APE, and thus can favor bacterial growth (Lechuga et al., 2016). However, there are differences between surfactant families regarding their degradability. For instance, and according to the obtained results, Triton X-100 (APE) is less readily biodegradable than SDS (alkylbenzenesulphonate), which is in agreement with previous observations (Okpokwasili and Olisa, 1991). The linear structure of SDS, compared to the branched and the ethoxylated chain of Triton X-100, may be among the reasons that explain its higher biodegradability (Zeng et al., 2007).

The inability of *S. obliquus*–associated bacteria to degrade a surfactant does not imply that such surfactant cannot be biodegraded by bacteria. For example, Triton X-100 has shown to be biodegradable by different *Pseudomonas* strains isolated from places that had been polluted with surfactants (Chen et al., 2005). But in that study the *Pseudomonas* strains were selectively cultivated in Triton X-100-enriched medium before the biodegradation test. Adaptation of the microorganisms to the surfactant used as sole carbon source is an important aspect and might contribute to the reported biodegradability (Jurado et al., 2013). However, the results obtained using non-adapted bacteria allowed to differentiate the susceptibility of the different surfactants to microbial degradation under the experimental conditions assessed.

4.3.4. Surfactants toxicity

The toxicity of the surfactants to *C. sorokiniana*, *N. gaditana* and *S. obliquus* was studied by analyzing the effect of the surfactants on microalgal growth and photosynthetic efficiency (**Fig. 4.5**). A surfactant concentration was considered toxic when a 10% reduction (dashed line in **Fig. 4.5**), or higher, was found in optical density and/or in the maximal photosynthetic efficiency of Photosystem II of the algal cultures with respect to the control cultures without surfactants after 72 hours. The surfactant concentrations tested were chosen in the range of those around the maximum non-toxic and the minimum toxic values. The growth and photosynthetic efficiency inhibition values for all the conditions assessed are enlisted in **Table 4.3**.

The toxicity results, considering the surfactant concentrations used in weight per volume units, showed different sensitivity of each microalga to a given surfactant. In general, the highest tolerance to the evaluated surfactants was found for *S. obliquus* followed by *C. sorokiniana,* while the most sensitive strain was *Nannochloropsis gaditana* according to the surfactant concentrations at which similar toxic effects were observed for the three species. Inter-species and intra-species variability have been reported in surfactant toxicity (Ivanković and Hrenović, 2010). This indicates that the toxicity of a single surfactant is highly specific, not only for the particular chemical compound but also for the microalgal species tested. The reason behind the species-dependent toxic effects of a certain surfactant might lay in the differences in algal cell wall composition among species. As example, *Chlorella vulgaris* showed a higher sensitivity to surfactants like Triton X-100 than *Chlorella emersonii*, since the former lacks the trilaminar outer wall composed of non-hydrolyzable macromolecules that is present in the cell wall of the latter (Corre et al., 1996).

According to their toxic effects on the selected microalgae, the evaluated surfactants could be divided in two main groups. The two natural surfactants (BSA and Saponin), the two Pluronics (F68 and P84), and Tween 20 showed the lowest levels of toxicity to the tested microalgae species. On the opposite side, the ionic surfactants (CTAB and SDS), both Tergitols (NP9 and TMN6) and Triton X-100 exhibited the highest levels of toxicity to both microalgae.

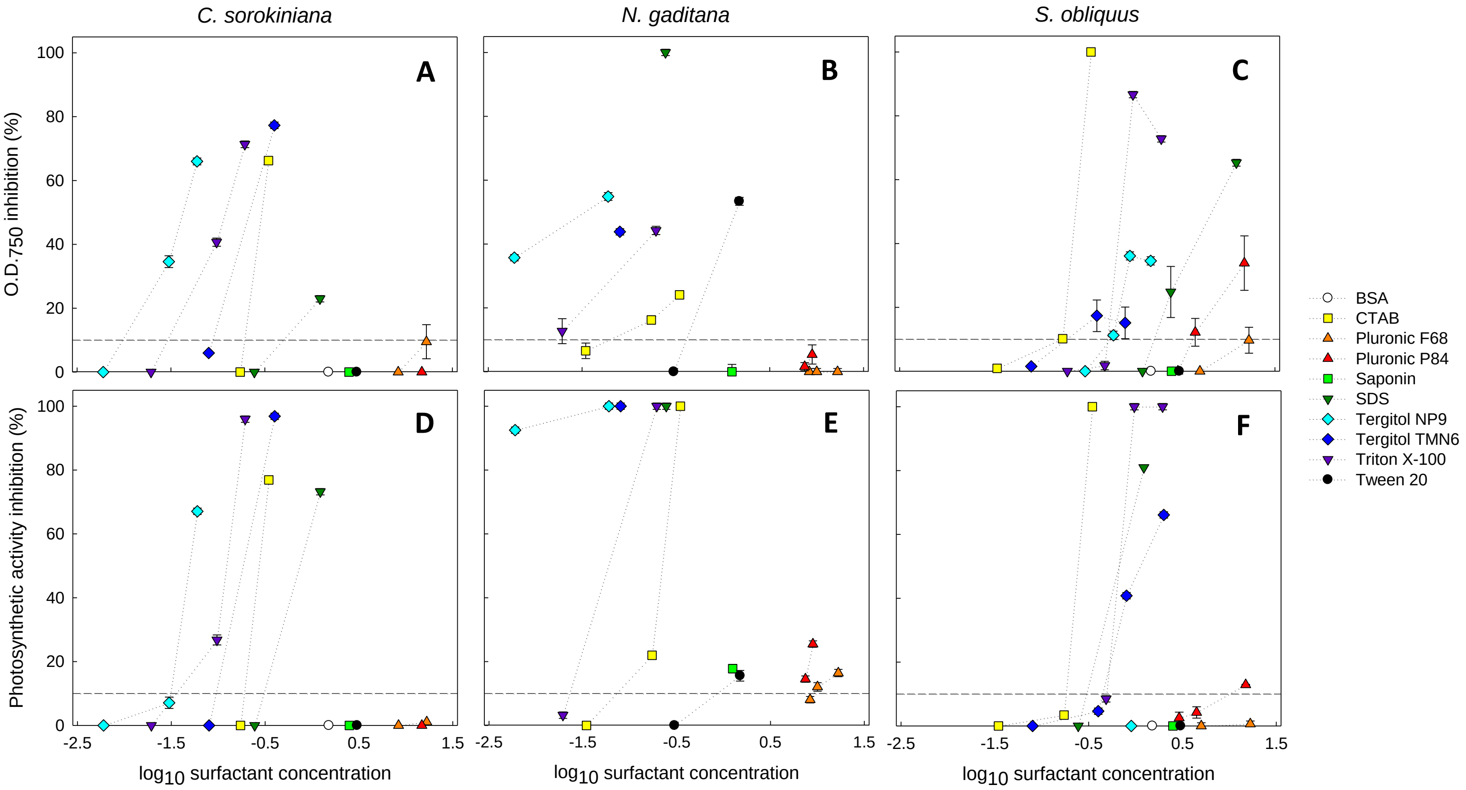


Figure 4.5. Surfactants concentration-response curves for (A, D) *Chlorella sorokiniana,* (B, E) *Nannochloropsis gaditana* and (C, F) *Scenedesmus obliquus* after 72 hours of exposure in liquid cultures. The ordinate indicates (A-C) the percentage of inhibition in the biomass growth (represented as optical density at 750 nm) and (D-F) in the maximal photosynthetic efficiency of Photosystem II (F_v/F_m) in dependence of the logarithm of the surfactant concentration used in grams per liter. The dashed line indicates the 10% inhibition threshold.

Table 4.3. Growth and photosynthetic efficiency inhibition values in terms of optical density at 750 nm inhibition (O.D., %) and inhibition of the maximal photosynthetic efficiency of Photosystem II (Fv/Fm, %) after 72 h of cultivation of *Chlorella sorokiniana Nannochloropsis gaditana* and *Scenedesmus obliquus* with the different surfactants. Negative inhibition values are shown as zero. Cultures in which measurements were not possible due to high turbidity are indicated with a minus symbol (-). N/A: not assessed. Additional inhibition values obtained in 6-well plates set-up are indicated with an asterisk (*).

		C. sorokiniana		***N. gaditana***		***S. obliquus***	
Surfactant	**Surfactant concentration (CMC)**	**O.D. inhibition (%)**	**F_v/F_m inhibition (%)**	**O.D. inhibition (%)**	**F_v/F_m inhibition (%)**	**O.D. inhibition (%)**	**F_v/F_m inhibition (%)**
BSA	1	N/A	N/A	N/A	N/A	0.00 ± 0.00	0.00 ± 0.00
	5	N/A	N/A	N/A	N/A	0.00 ± 0.00	0.00 ± 0.00
	10	N/A	N/A	N/A	N/A	0.00 ± 0.00	0.00 ± 0.00
	25	N/A	N/A	N/A	N/A	0.00 ± 0.00	0.00 ± 0.00
	50	0.00 ± 0.00	0.00 ± 0.00	N/A	N/A	0.00 ± 0.00	0.00 ± 0.00
CTAB	0.1	0.00 ± 0.00	0.00 ± 0.00	6.55 ± 2.44	0.00 ± 0.00	0.90 ± 1.28	0.00 ± 0.00
	0.5	0.00 ± 0.00	0.00 ± 0.00	16.21 ± 1.00	21.95 ± 1.00	10.18 ± 1.28	3.39 ± 1.00
	1	66.23 ± 1.07	76.92 ± 1.00	24.07 ± 1.00	100.00 ± 1.00	-	100.00 ± 1.00
Pluronic F68	1	0.00 ± 0.00	0.00 ± 0.00	N/A	N/A	N/A	N/A
	5	0.00 ± 0.00	0.00 ± 0.00	N/A	N/A	N/A	N/A
	10	N/A	N/A	N/A	N/A	0.00 ± 0.00	0.00 ± 0.00
	15	*0.00 ± 0.00	*0.00 ± 0.00	N/A	N/A	0.00 ± 0.00	0.00 ± 0.00
	25	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	N/A	N/A
	30	0.00 ± 0.00	0.00 ± 0.00	* 0.00 ± 0.00	* 12.06 ± 1.34	N/A	N/A
	50	9.49 ± 5.34	1.08 ± 1.00	* 0.00 ± 0.00	* 16.42 ± 1.14	9.69 ± 4.05	0.63 ± 0.90

Table 4.3. *(Continuation).*

		C. sorokiniana		***N. gaditana***		***S. obliquus***	
Surfactant	**Surfactant concentration (CMC)**	**O.D. inhibition (%)**	**F_v/F_m inhibition (%)**	**O.D. inhibition (%)**	**F_v/F_m inhibition (%)**	**O.D. inhibition (%)**	**F_v/F_m inhibition (%)**
Pluronic P84	1	*0.00 ± 0.00	*0.00 ± 0.00	N/A	N/A	N/A	N/A
	5	0.00 ± 0.00	0.00 ± 0.00	N/A	N/A	N/A	N/A
	10	N/A	N/A	N/A	N/A	0.00 ± 0.00	2.53 ± 1.79
	15	*0.00 ± 0.00	*0.00 ± 0.00	N/A	N/A	12.17 ± 4.34	4.23 ± 1.79
	25	*0.00 ± 0.00	*0.00 ± 0.00	1.57 ± 4.32	14.52 ± 1.00	N/A	N/A
	30	N/A	N/A	5.39 ± 16.10	25.49 ± 1.02	N/A	N/A
	50	0.00 ± 0.00	0.00 ± 0.00	N/A	N/A	33.85 ± 8.53	12.9 ± 0.12
Saponin	1	N/A	N/A	N/A	N/A	0.00 ± 0.00	0.00 ± 0.00
	5	N/A	N/A	N/A	N/A	0.00 ± 0.00	0.00 ± 0.00
	10	*0.00 ± 0.00	*0.00 ± 0.00	N/A	N/A	0.00 ± 0.00	0.00 ± 0.00
	25	*0.00 ± 0.00	*0.00 ± 0.00	0.00 ± 0.00	17.78 ± 1.35	0.00 ± 0.00	0.00 ± 0.00
	50	0.00 ± 0.00	0.00 ± 0.00	N/A	N/A	0.00 ± 0.00	0.00 ± 0.00
SDS	0.1	0.00 ± 0.00	0.00 ± 0.00	100.00 ± 1.00	100.00 ± 1.00	0.00 ± 0.00	0.00 ± 0.00
	0.5	-	73.21 ± 1.00	-	100.00 ± 1.00	-	80.95 ± 1.00
	1	N/A	N/A	N/A	N/A	24.82 ± 53.80	-
	5	N/A	N/A	N/A	N/A	65.31 ± 1.07	-

Table 4.3. *(Continuation).*

		C. sorokiniana		***N. gaditana***		***S. obliquus***	
Surfactant	**Surfactant concentration (CMC)**	**O.D. inhibition (%)**	**F_v/F_m inhibition (%)**	**O.D. inhibition (%)**	**F_v/F_m inhibition (%)**	**O.D. inhibition (%)**	**F_v/F_m inhibition (%)**
Tergitol NP9	0.1	0.00 ± 0.00	0.00 ± 0.00	35.71 ± 1.00	92.50 ± 1.00	N/A	N/A
	0.5	34.58 ± 1.84	7.09 ± 1.77	N/A	N/A	N/A	N/A
	1	65.99 ± 1.08	67.07 ± 1.00	54.88 ± 1.21	100.00 ± 1.00	0.00 ± 0.00	0.00 ± 0.00
	5	N/A	N/A	N/A	N/A	0.00 ± 0.00	0.00 ± 0.00
	10	N/A	N/A	N/A	N/A	11.24 ± 1.31	0.00 ± 0.00
	15	N/A	N/A	N/A	N/A	36.11 ± 1.27	0.00 ± 0.00
	25	N/A	N/A	N/A	N/A	34.56 ± 1.28	0.00 ± 0.00
Tergitol TMN6	0.1	5.97 ± 0.00	0.00 ± 0.00	43.83 ± 1.00	100.00 ± 1.00	0.00 ± 0.00	0.00 ± 0.00
	0.5	77.29 ± 1.03	96.85 ± 1.00	N/A	N/A	17.36 ± 4.95	4.62 ± 1.00
	1	N/A	N/A	N/A	N/A	15.14 ± 4.95	40.79 ± 1.00
	2.5	N/A	N/A	N/A	N/A	7.08 ± 1.29	66.10 ± 1.00
Triton X-100	0.1	0.00 ± 0.00	0.00 ± 0.00	12.69 ± 3.91	3.33 ± 1.00	N/A	N/A
	0.5	40.68 ± 1.28	26.77 ± 1.56	N/A	N/A	N/A	N/A
	1	71.33 ± 1.05	95.97 ± 1.00	44.24 ± 1.30	100.00 ± 1.00	0.00 ± 0.00	9.68 ± 1.00
	2.5	N/A	N/A	N/A	N/A	1.77 ± 1.33	8.47 ± 1.00
	5	N/A	N/A	N/A	N/A	86.65 ± 1.00	100.00 ± 1.00
	10	N/A	N/A	N/A	N/A	72.79 ± 1.00	100.00 ± 1.00

Table 4.3. *(Continuation).*

		C. sorokiniana		***N. gaditana***		***S. obliquus***	
Surfactant	**Surfactant concentration (CMC)**	**O.D. inhibition (%)**	**F_v/F_m inhibition (%)**	**O.D. inhibition (%)**	**F_v/F_m inhibition (%)**	**O.D. inhibition (%)**	**F_v/F_m inhibition (%)**
Tween 20	0.1	N/A	N/A	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	0.5	0.00 ± 0.00	0.00 ± 0.00	N/A	N/A	N/A	N/A
	2.5	0.00 ± 0.00	0.00 ± 0.00	N/A	N/A	0.00 ± 0.00	0.00 ± 0.00
	5	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	10	0.00 ± 0.00	0.00 ± 0.00	N/A	N/A	N/A	N/A
	15	0.00 ± 0.00	0.00 ± 0.00	N/A	N/A	N/A	N/A
	20	0.00 ± 0.00	0.00 ± 0.00	N/A	N/A	N/A	N/A
	25	0.00 ± 0.00	0.00 ± 0.00	53.37 ± 1.23	15.56 ± 1.67	0.00 ± 0.00	0.00 ± 0.00
	30	0.00 ± 0.00	0.00 ± 0.00	N/A	N/A	N/A	N/A
	50	0.00 ± 0.00	0.00 ± 0.00	N/A	N/A	0.00 ± 0.00	0.00 ± 0.00

In general, surfactant toxicity decreases according to the following order: cationic>anionic>non-ionic surfactants (Lewis, 1990). Consistently with this general statement, high toxicity was found for the ionic surfactants (CTAB and SDS). Within the non-ionic surfactants tested, the APE surfactants (Tergitol NP9 and Triton X-100) showed high toxicity, which can be related to the benzyl group contained as part of their structures. The APE surfactants have very similar structures and the main difference between them is that while Tergitol NP9 has a linear alkyl chain of nine carbon atoms, Triton X-100 has a shorter branched alkyl group. In general, lower concentrations of Tergitol NP9 were needed to produce the same toxic effects as with Triton X-100 (**Fig. 4.5**). According to previous studies, the role of the hydrophobic alkyl chain is important in surfactant toxicity (Fernández-Serrano, 2014). They can interact with lipid parts of the biological membranes (including the thylakoids) (Kráľová and Šeršeň, 2012). The longer the carbon chain, the greater the interaction with the cell membranes (Ivanković and Hrenović, 2010; Yoo et al., 2016), which possibly explains the higher toxicity of Tergitol NP9 compared to that of Triton X-100.

Moreover, among the surfactants with FAE structure, Tergitol TMN6 showed much higher toxicity than the poloxamers (Pluronic F68 and P84). Surfactants with the same electrostatic character (e.g. non-ionic) but different chemical structure, may exhibit different toxicity to a given microalga species. For instance, this is the case for both Pluronics, which exert different toxic effects on *S. obliquus* (**Fig. 4.5**), Pluronic F68 being less toxic than Pluronic P84. Poloxamers are copolymers of ethylene oxide (hydrophilic) and propylene oxide (lipophilic) blocks. Their toxicity has been reported to be proportional to their lipophilic character (Johnston and Miller, 1985) which is in agreement with our findings; propylene oxide blocks comprise only 20% of the Pluronic F68 molecule whereas Pluronic P84 contains 60% of hydrophobic blocks.

In general, surfactants primarily affect the growth, motility and photosynthetic activity of algae (Rebello et al., 2014). In our experiments the surfactants affected similarly the two parameters investigated: algal growth and photosynthetic activity. In general, certain concentration of a particular surfactant resulted in the inhibition of both parameters to a similar extent. However, in some cases the surfactant affected one parameter further. As example, high concentrations of

Pluronics exhibited inhibitory effects on the growth of *S. obliquus* (**Fig. 4.5C**) while its photosynthetic activity was hardly affected (**Fig. 4.5F**).

Next to algal growth and photosynthetic efficiency, morphological changes in the colony-forming strain *S. obliquus* were observed under the optical microscope when incubated at high concentrations (100 CMC) of the surfactant Saponin (**Fig. 4.6**). In these cultures, the algal colonies disintegrated and the cells became rounder than those forming the colonies in the surfactant-free cultures. It has been reported that surfactants can produce morphological changes in the cells (Azizullah et al., 2011). Besides, Saponin promoted bacterial growth in the algal cultures, and the changes observed in the algal colonies might also be due to the high bacterial concentration in the algal cultures. As example, high competitive pressure exerted by large populations of other microorganisms such as *Microcystis aeruginosa* has shown to inhibit the formation of colonies of *S. obliquus* (Zhu et al., 2015).

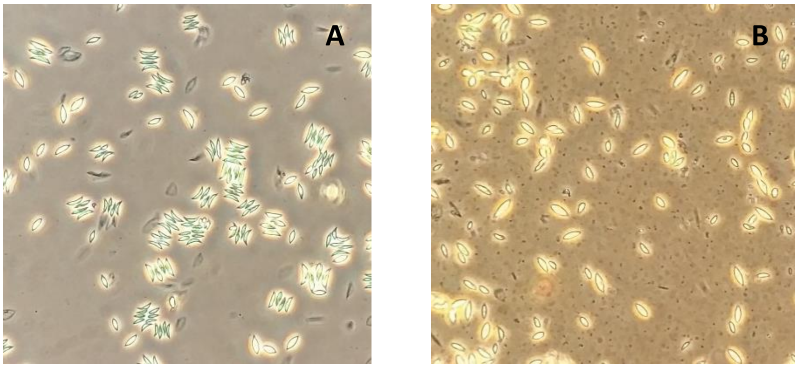


Figure 4.6. Optical microscope images of *Scenedesmus obliquus* after five days of cultivation: (A) with no surfactant added and (B) with Saponin at 100 CMC.

4.3.5 Surfactant selection

To select the most appropriate surfactant for the liquid foam-bed photobioreactor, the foaming properties, microalgae partitioning towards the foam, biodegradability and toxicity were evaluated together.

The optimal surfactant for the liquid foam-bed photobioreactor should be capable of forming large foam volumes, with small and uniform bubbles. Small bubbles are relevant for increased mass transfer in the foam-bed photobioreactor due to the increased surface area. Further advantage of small bubbles is their increased particle flotation efficiency, possibly resulting in an increased algae partitioning towards the foam phase (Al-Shamrani et al., 2002). On the one hand, the foam formed should not be too stable (>2 h, e.g. CTAB and Saponin at 5 CMC) in order to facilitate foam break-up. Fast destabilization, on the other hand, as observed with Tween 20 and Tergitol TMN6, might result in foam collapse already while the foam is rising in the reactor, resulting in heterogeneous, coalescing foams. This would be especially problematic at low gas flow rates or high foam-bed photobioreactors. Besides, the liquid content of the foam should be intermediate in order to achieve increased biomass concentrations compared to conventional photobioreactors, while still maintaining high ground-areal and volumetric productivities.

Altogether, the following ranges were identified for the foaming properties assessed: the liquid holdup of surfactants should be between 5-10%, the foam volume half-life should be between 25 and 120 minutes, the bubble radius should be smaller than 0.4 mm, and surfactants should show a constant foam up-flow speed during at least 20 cycles. As shown in **Figure 4.1**, surfactants with a higher liquid holdup than 5% were BSA, Pluronic F68, Tergitol TMN6, Tween 20, Pluronic P84 and SDS at 5 CMC. Surfactants with intermediate foam stabilities were BSA, Tergitol NP9, Pluronic F68, Pluronic P84 and Triton X-100. The bubble size was too large for Saponin, all other surfactants fell in the good range. The protein BSA and CTAB did not show sufficient stability against foaming.

For the liquid foam-bed photobioreactor, maximal partitioning of microalgae to the foam phase is preferred. Elevated algae concentrations in the foam phase result in an increased fraction of algal cells exposed to illumination, thus contributing to higher growth rates and reactor productivity. Non-ionic and anionic surfactants led to generally low algal partitioning values and the differences between them were relatively small. Nevertheless, apart from the cationic surfactant CTAB, Triton X-100, BSA and Saponin were considered to show the best performances.

Regarding surfactant biodegradability, the selected surfactant must not be easily degraded by the bacterial populations that are naturally associated to the algal culture. The use of a readily biodegradable foam stabilizing agent would imply a relatively rapid increase of bacterial populations in the algal cultures, together with the necessity of continuous surfactant addition in order to compensate its consumption and to maintain its concentration constant in the liquid foam-bed photobioreactor. In this study, 3 of the 10 surfactants tested (BSA, Saponin and Tween 20) were biodegraded at surfactant concentrations higher than 1 CMC. Considering the foam requirements, the surfactant concentration to be used in a liquid foam-bed photobioreactor needs to be above 1 CMC and, therefore, these surfactants were discarded for their use.

The last requisite of a surfactant to be employed for algal cultivation in foam is to be non-toxic for the alga cultivated. In general, most of the surfactants showed toxicity below 10 CMC (**Table 4.4**). The cationic surfactant CTAB, the anionic surfactant (SDS), and both Tergitols resulted as the most toxic surfactants for the three strains if compared in terms of CMC. In this sense, the criterion for a non-toxic surfactant is the absence of toxic effects (no reduction of either the growth rate or the photosynthetic efficiency) at the concentration of 10 CMC. According to this, only both Pluronics, BSA, Saponin and Tween 20 fit the requirement. However, for the cultivation of other strains, the tolerance of the three species investigated to the given surfactants cannot be extrapolated to others and should be specifically studied.

Table 4.4. Maximum surfactant concentration tested with no toxic effects for the microalgal strains *Chlorella sorokiniana*, *Nannochloropsis gaditana* and *Scenedesmus obliquus*, expressed in function of their CMC values. Toxicity of BSA to *N. gaditana* was not assessed (N/A).

	Experimental maximum non-toxic surfactant concentration (CMC)									
	BSA	**CTAB**	**Pluronic F68**	**Pluronic P84**	**Saponin**	**SDS**	**Tergitol NP9**	**Tergitol TMN6**	**Triton X-100**	**Tween 20**
C. sorokiniana	50	0.5	50	50	50	0.1	0.1	0.1	0.1	50
N. gaditana	N/A	0.1	<30	<25	<25	<0.1	<0.1	<0.1	<0.1	5<x<25
S. obliquus	50	0.1	50	10	50	0.1	5	0.1	2.5	50

In order to summarize the main findings, surfactants showing acceptable properties with respect to each individual criterion were indicated with a '+' in **Table 4.5**. Considering all properties together, the poloxameric surfactants (Pluronic F68 and P84) were the best candidates investigated for the operation of the liquid foam-bed photobioreactor, due to their good foaming properties, resistance to biodegradation, moderate algae partitioning, and low toxicity. The most important limitation of these surfactants lies in the fact that they have low P_x^{foam} values (i.e. partitioning towards the foam). By minimizing the amount of bulk liquid in an operational foam-bed photobioreactor, the absolute amount of algae in the relatively dark bulk liquid can be minimized, thus the majority of the microalgae still residing in the foam phase. Moreover, the algae partitioning towards the foam formed by poloxamers can be further improved in the foam-bed photobioreactor by minimizing the amount of bulk liquid and bubbles sizes (Hu et al., 2007; Hunter et al., 2008; Sobczuk et al., 2006) or by increasing the surfactant concentration (Chen et al., 1998) and the aeration rates (Phoochinda and White, 2003). From the two tested poloxamers, Pluronic F68 was selected as the best surfactant since it performed slightly better in terms of foaming, algae partitioning and toxicity.

Table 4.5. Summary of the surfactant selection experiments. The "+" indicates that a certain surfactant has acceptable properties regarding the corresponding criterion. Foaming properties: foam volume half-life time (A), liquid holdup in the foam (B), bubble size of the foam (C) and surfactant stability (D).

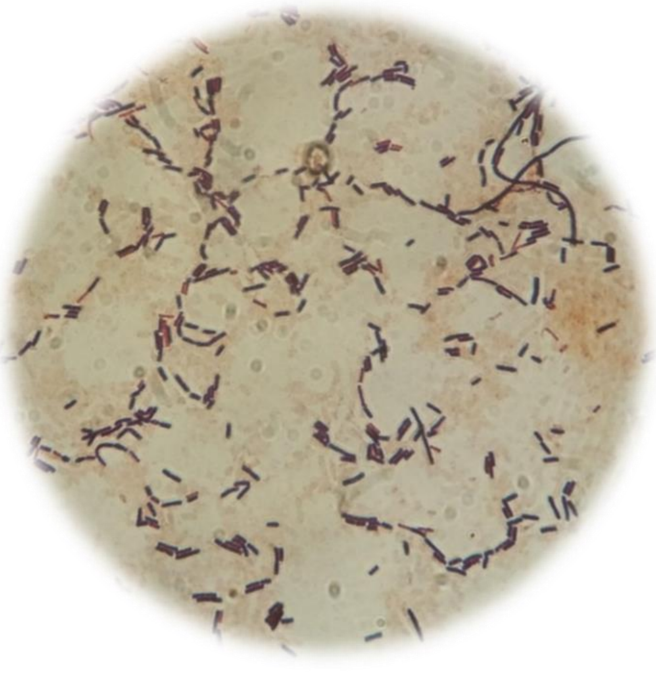
Surfactant	**Foaming properties**				**Algae partitioning**	**Resistance to biodegradability**	**Toxicity**
	A	**B**	**C**	**D**			
BSA	+	+	+	-	+	-	+
CTAB	-	-	+	-	+	+	-
Pluronic F68	+	+	+	+	-	+	+
Pluronic P84	+	+	+	+	-	+	+
Saponin	-	-	-	+	+	-	+
SDS	-	+	+	+	-	-	-
Tergitol NP9	+	-	+	+	-	-	-
Tergitol TMN6	-	+	+	+	-	-	-
Triton X-100	+	-	+	+	+	+	-
Tween 20	-	-	+	+	-	-	+

4.4. Conclusions

This study was aimed at selecting an optimal surfactant for the operation of a foam-bed photobioreactor. Ten potential candidates were tested in terms of their foaming properties, microalgal partitioning towards the foam, biodegradability and toxicity using *Chlorella sorokiniana, Nannochloropsis gaditana* and *Scenedesmus obliquus,* whose potential to be cultivated in foam was previously described. According to the results, the poloxameric surfactants (Pluronic F68 and P84) exhibited the best properties, and in particular, Pluronic F68 showed the best performance. This surfactant showed constant foamability during repetitive foaming cycles and at 5 CMC concentration it formed foams with intermediate stability (54 minutes' half-life), high liquid holdup (8% at 400 mL min^{-1} superficial gas velocity), and small bubble size (radius of 0.17 mm) which are optimal for the operation of the liquid foam-bed photobioreactor. Besides, it is hardly biodegradable by the bacteria naturally associated to microalgae, therefore the surfactant concentration in the reactor is not reduced due to bacterial consumption. The relatively low microalgae partitioning towards the foam phase formed by Pluronic F68 (67% for *C. sorokiniana* and 47% for *S. obliquus*) could be compensated by optimizing the operation and design of the liquid foam-bed photobioreactor. Finally, it showed the lowest toxicity; growth inhibition was not observed for any of the three microalgal strains tested until 50 CMC. Thus, Pluronic F68 is a promising surfactant for its utilization in a liquid foam-bed photobioreactor.

Chapter 5

Biodegradability of BSA, Saponin and Tween 20 by *Scenedesmus obliquus*-associated bacteria



Sections of this Chapter will be submitted as:

Vázquez M, Castilla JC, Vílchez C, Cuaresma M. Biodegradability of BSA, Saponin and Tween 20 by *Scenedesmus obliquus*-associated bacteria

5.1 Abstract

The ability of *Scenedesmus obliquus*-associated bacteria to biodegrade the surfactants BSA, Saponin and Tween 20 has been proved. However, the effects of these biodegradable surfactants on the behavior of the whole microbial community of microalgal cultures (i.e. microalgal and bacterial populations) have not been determined. In this Chapter, the biodegradability of the surfactants BSA, Saponin and Tween 20 was further investigated in liquid cultures of *S. obliquus* at concentrations up to 50 CMC. The effect of the surfactants over the growth of *S. obliquus* and the bacterial populations present in the cultures were studied. The three surfactants showed to promote both bacterial and algal growth in the range of concentrations evaluated, although differences among the three surfactants were found regarding their efficiency to sustain bacterial growth.

Besides, the biodegradability of Saponin was further investigated in algal-free cultures of *S. obliquus*-associated bacteria, which showed to be a consortium formed by at least 4 different bacterial strains, including both Gram positive and Gram-negative bacteria. Saponin showed to enhance the growth of these bacteria and to sustain it even when other nutrients were lacking, which suggests that Saponin can be used as energy and nutrients source. The degradation and consumption of Saponin by *S. obliquus*-associated bacteria was also confirmed by the decrease of the foaming capacity of Saponin-added bacterial cultures. The biodegradable character of BSA, Saponin and Tween 20 reduce their suitability to be used in a liquid-foam photobioreactor since they would not be able to maintain a stable foaming.

5.2 Introduction

In the previous Chapter of this Thesis (Chapter 4; Janoska and Vázquez et al., 2018) the selection of a potentially suitable surfactant for microalgae cultivation in surfactant-stabilized foam was performed. This was conducted by assessing ten surfactants according to four criteria, which were considered to be crucial for an optimal microalgae cultivation process in a liquid foam-bed photobioreactor (LF-PBR). It was determined that a suitable surfactant should fit the following requirements: (i) it should have good foaming properties, (ii) it should drag-up the microalgae in the foam formed, (iii) it should not be rapidly biodegradable, and (iv) it should not be toxic for microalgae.

In particular, the biodegradability of the surfactant was determined to be crucial due to two different reasons. First, the use of a biodegradable surfactant in a LF-PBR could result in the decrease of the foaming capacity of the surfactant-added culture. The biodegradation of the surfactant could, therefore, have an impact on the stability of the surfactant concentration in the reactor and it could result in the necessity of continuous addition of surfactant in order to maintain an optimal surfactant concentration in the system. The biodegradation of a surfactant can be either partial or complete (Ying, 2006). In the latter case, it is obvious that the foaming capacity of the surfactant-added culture would decrease as the surfactant is being degraded. Hence, the biodegradability of a surfactant was considered to be a drawback for its application in a LF-PBR. Secondly, microalgal cultures generally contain other associated microorganisms including bacteria. Obtaining axenic microalgal cultures is an onerous task (Ramanan et al., 2016) and the scale-up of microalgal cultures to large scale under axenic conditions would be extremely difficult and unpractical. These bacterial populations that are naturally associated to microalgae are species specific and their interactions with the microalgal cells can range from beneficial to detrimental to algal growth (Fuentes et al., 2016). In the worst case, bacteria can act as parasites for microalgae. For instance, Wang and co-workers (2010) studied the effect of increasing the bacteria concentration in the phycosphere of the dinoflagellate *Alexandrium tamarense*. The term "phycosphere" refers to "the zone that may exist extending outward from an algal cell or colony for an undefined distance, in which the bacterial growth is stimulated by the extracellular products of the alga", according to the definition of Bell and Mitchell (1972). Wang and co-workers (2010) found that adding bacterial culture medium to the dinoflagellate culture altered the balance between algal and bacterial populations in the culture, which in turn resulted in algal cell lysis. However, bacteria can also positively influence microalgal growth and even modify their biochemical composition. It is known that bacteria can enhance microalgal growth through the production of growth-promoting factors such as indole-3-acetic acid, vitamins and siderophores, among others (Fuentes et al., 2016). Moreover, these bacteria-algal interactions can be one-way (commensalism) or bidirectional (mutualism). As example, *Azospirillum brasilense* was found to promote the growth of *Chlorella vulgaris* and *Chlorella sorokiniana* when co-immobilized and grown in wastewater (De-Bashan et al., 2004), but also an enhanced accumulation of starch and carbohydrates has been observed for these two *Chlorella* strains (Choix et al., 2012).

Considering the varied effects of the bacteria-microalga interactions summarized above, the presence of an additional carbon and energy source for the bacteria in the microalgal culture (i.e. a biodegradable surfactant) can have unpredictable effects over the culture, and further research in this sense is necessary.

In Chapter 3, *Scenedesmus obliquus* showed to be a promising microalgal strain to be cultivated in a LF-PBR and in Chapter 4 the biodegradability of 10 surfactants was assessed using *S. obliquus*-associated bacteria. Considering that *S. obliquus* has been observed to grow in symbiosis with bacteria (Ferreira et al., 2017), and that the co-cultivation of this microalga and selected bacterial strains could result in enhanced microalgal growth (Wang et al., 2015b), it was decided to further study the effects of these biodegradable surfactants on the growth of both the bacteria and the microalga.

From the studies performed in the previous Chapter of this Thesis, the surfactants BSA, Saponin and Tween 20 were found to be easily biodegradable. In that study, the biodegradability of these surfactants was investigated at concentrations of 1 and 10 CMC, since the surfactant concentration employed in a LF-PBR was expected to be in this range. Nevertheless, in order to get a greater insight on the effect of a biodegradable surfactant on the microbial populations of a microalgal culture, a wider range of surfactant concentrations should be studied.

Biodegradable surfactants are, in general, less toxic to microalgae. This can be exemplified by the most easily biodegradable surfactants found in the previous Chapter, which were BSA, Saponin and Tween 20. These surfactants were, together with the Pluronic surfactants, the best tolerated by the three microalgal species tested. Considering that promoting bacterial growth may have a positive effect on microalgal growth, the use of a biodegradable surfactant in surfactant-stabilized foam-based cultures might in turn enhance microalgal growth. However, very little is known regarding the performance of microalgal cultures with the addition of biodegradable surfactants. Some questions still unanswered include: (i) whether bacteria naturally associated to the algal cultures are able to degrade these compounds either partially or completely, (ii) how an increase of the bacterial populations would affect microalgal growth and the performance of the microalgal culture as a whole, and (iii) to which extent this can affect the foaming capacity of the cultures. Therefore, more research in this sense is deemed necessary.

This Chapter aims at studying the impact of the addition of three biodegradable surfactants (BSA, Saponin, Tween 20) on the microbial populations of *Scenedesmus obliquus* cultures, including both the microalga and the bacterial associated populations. Besides, a greater insight on the biodegradation of Saponin by *S. obliquus*-associated bacteria is provided by studying the effect of the biodegradation of Saponin on its foaming capacity using algal-free bacterial cultures.

5.3 Results and discussion

5.3.1 *Scenedesmus obliquus*-associated bacteria

The bacteria present in *Scenedesmus obliquus* cultures were proved to be responsible for the biodegradation of some of the surfactants tested (Chapter 4, **Section 4.3.3**). In order to characterize partially these bacteria, they were isolated from a *S. obliquus* culture by streaking aliquots of the culture broth on Tryptone Glucose Agar (TGA) plates. Gram-staining of the bacteria isolated from *S. obliquus* cultures showed that this consortium was composed of more than one strain, including Gram-negative and Gram-positive bacteria (**Fig. 5.1**). Moreover, the differences in the morphological features of the colonies grown on the agar plates allowed to identify the presence of at least four bacterial species. Hence, more than one bacterial strain may be responsible for the previously observed biodegradation of the surfactants tested. In this sense, other studies on the bacterial communities of *S. obliquus* cultures have found bacteria from at least ten different orders (Greses et al., 2017), and twenty-five different species were found in *S. obliquus* cultures by Wang et al. (2015b), among which five were able to grow using the nutrients secreted by the alga.

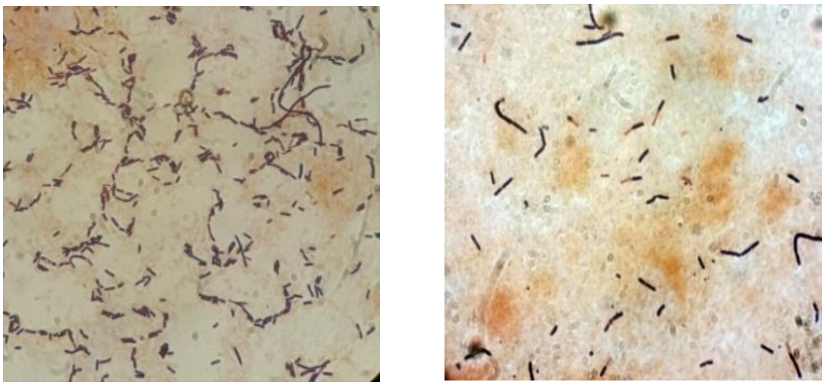


Figure 5.1. Optical microscope images of Gram-stained *Scenedesmus obliquus*-associated bacteria.

5.3.2. Surfactants biodegradation in algal cultures

The biodegradation of the surfactants BSA, Saponin and Tween 20 was further investigated in microalgal cultures in order to get a greater insight on the microalgae-bacteria interactions. In this study, biodegradation was investigated in *S. obliquus* cultures because this microalga showed to be the most tolerant to the surfactants evaluated (Chapter 4, **Section 4.3.4**); therefore its potential for microalgal cultivation in foam was considered the highest among the microalgae tested in this work, at the time of these experiments. The surfactants BSA, Saponin and Tween 20, together with SDS, showed to be the most easily biodegraded by *Scenedesmus obliquus*-associated bacteria at 1 and 10 CMC concentrations (Chapter 4, **Fig. 4.4**). In the experiments presented below, the biodegradation of the surfactants was investigated at concentrations up to 50 CMC. The surfactant SDS was not used in these experiments because of its high CMC value (see Materials and Methods, **Table 2.1**). To assess the biodegradation of this surfactant at 50 CMC, approximately 25 g L^{-1} SDS had to be added, which resulted in evident solubility and precipitation problems. Besides, the maximum SDS concentration tolerated by *S. obliquus* was already known to be 0.1 CMC, while BSA, Saponin and Tween were proved to be non-toxic at concentrations up to 50 CMC (Chapter 4, **Table 4.4**).

BSA, Saponin and Tween 20 were added to *S. obliquus* cultures at different concentrations: 1, 5, 10, 25 and 50 CMC (**Fig. 5.2**). Bacterial and algal growth in the presence of the biodegradable surfactants was measured in terms of optical density at 600 and 750 nm, respectively, since it is a rapid and commonly-used technique for estimating bacterial and microalgal concentrations in liquid cultures (Griffiths et al. 2011; Myers et al., 2013). The optical density at 600 nm was measured in the supernatant of microalgal broth samples after removing the algal biomass by gentle centrifugation. Prior measuring the optical density at 750 nm, the biomass pellet formed after centrifugation was resuspended in the same sample volume of fresh culture medium. Optical density was measured in this microalgal suspension, thereby preventing turbidity interferences due to high bacterial content. The evolution of bacterial and algal concentrations and the ratio between both populations in the algal cultures incubated with the different surfactants are shown in **Figure 5.3.**

Figure 5.2. Picture of *Scenedesmus obliquus* cultures after 2.8 days of cultivation with the addition of Tween 20, BSA and Saponin at concentrations from 1 to 50 CMC.

As can be seen in **Figure 5.3**, the three surfactants promoted both bacterial and algal growth in the range of concentrations tested. Bacterial and algal growth increased with increasing surfactant concentrations in all cases, especially above 10 CMC concentration. In addition, the photosynthetic efficiency of all the cultures remained unaffected during the experiment (data not shown), which restates the non-toxic nature of these surfactants to *S. obliquus.*

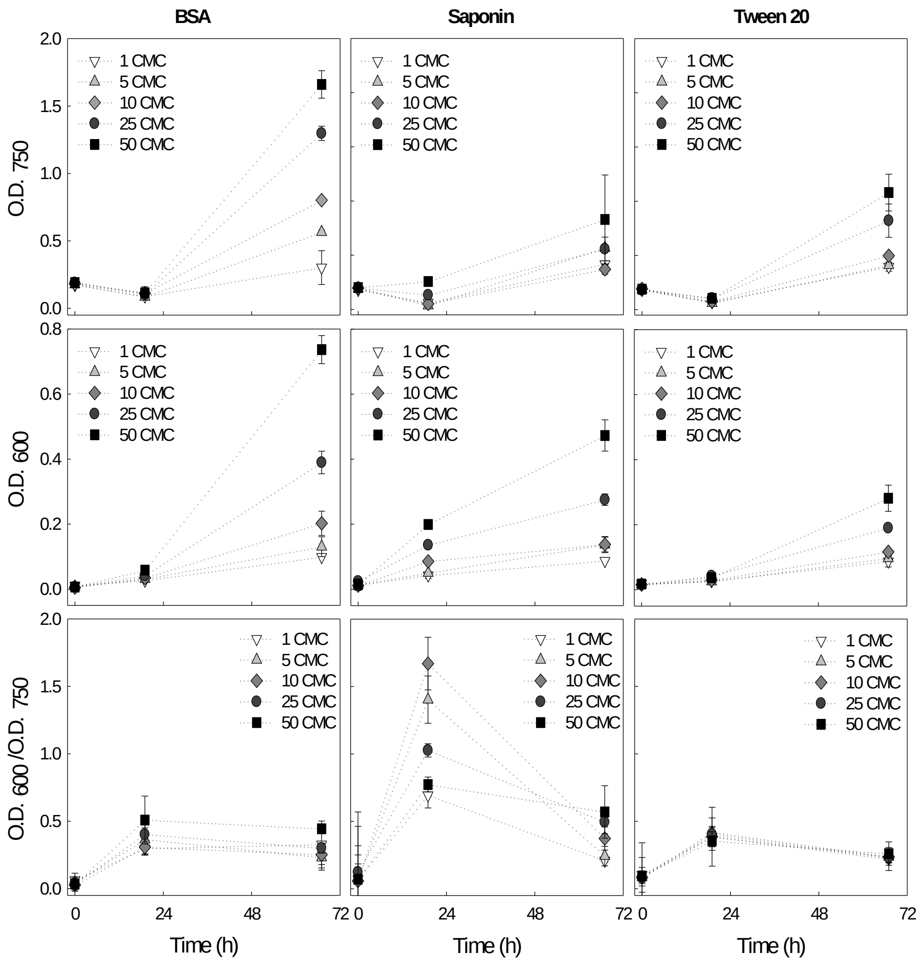


Figure 5.3. *Scenedesmus obliquus* (upper figures) and bacterial (middle figures) growth expressed as optical density at 750 nm (O.D.$_{750}$) and 600 nm (O.D.$_{600}$) respectively. Bacteria to microalgae ratio (lower figures) expressed as the ratio between the optical density at 600 and 750 nm (O.D.$_{600}$/O.D.$_{750}$). Cultures with BSA (left), Saponin (middle) and Tween 20 (right) at concentrations of 1, 5, 10, 25 and 50 CMC. The average values of duplicate cultures are shown, together with their corresponding standard deviations.

Besides, both the bacteria and the microalga showed a lag phase of at least 20 hours when cultivated at the different concentrations of the three surfactants, with the exception of the bacteria in Saponin cultures, which showed growth from the beginning of the experiment (**Fig. 5.3**). This resulted in a marked increase of the bacteria to algae ratio during the first day of cultivation in the presence of

Saponin. Microorganisms usually show a latency time to acclimate themselves to the new substrate (Jurado et al., 2013; Mølgaard et al. 2000), which are the surfactants in this case. Bacteria started growing earlier in Saponin-enriched cultures than in cultures with BSA and Tween 20. Differences in bacterial growth at different surfactant concentrations were already noticeable after 24 h of cultivation in cultures with Saponin, unlike in those with BSA and Tween 20. This possibly indicates that the associated changes in the bacterial populations (i.e. growth of the bacterial populations responsible for the surfactant degradation) or the changes in the bacterial metabolism to degrade the surfactants (e.g. gene expression and/or enzyme induction) were more complex for the uptake and utilization of BSA and Tween 20 than for Saponin. Moreover, the bacteria to algae ratio (i.e. $O.D._{600}/O.D._{750}$) increased in the presence of the three surfactants. The experimental cultivation conditions may have contributed to the general increase in this ratio, which was initially 0.071±0.027. Unlike the experimental cultures, the inoculum was obtained from a culture that was bubbled with CO_2-enriched air, which favors algal growth over bacterial growth. However, the surfactants influenced the bacteria to algae ratio differently. For BSA-added cultures, slightly higher ratios were found with increased BSA concentrations. In these cultures, the ratio increased in the first 24 hours of cultivation and remained constant until the end of the experiment. As aforementioned, the addition of Saponin resulted in a steeply increased of the bacteria to alga ratio in the first day of cultivation. This increase was especially steep at intermediate Saponin concentrations (i.e. 5-25 CMC). Finally, the addition of Tween 20 resulted in slightly higher bacteria to algae ratios, but this increase was similar over the range of concentrations tested.

In order to get a better insight of the effect of each biodegradable surfactant on the bacterial and algal populations, the increment in optical density at 600 and 750 nm, and the ratio between these two values were calculated at the end of the experiment and are shown in **Figures 5.4**, **5.5** and **5.6**. As previously explained, the surfactants have varied CMC values and chemical compositions, hence the carbon supplied by each surfactant differed when added at the same concentration in CMC units (**Table 4.2**). Subsequently, the X-axis of **Figures 5.4, 5.5** and **5.6** is expressed as the decimal logarithm of the milligrams of carbon added to each culture, calculated from the carbon content of the surfactants.

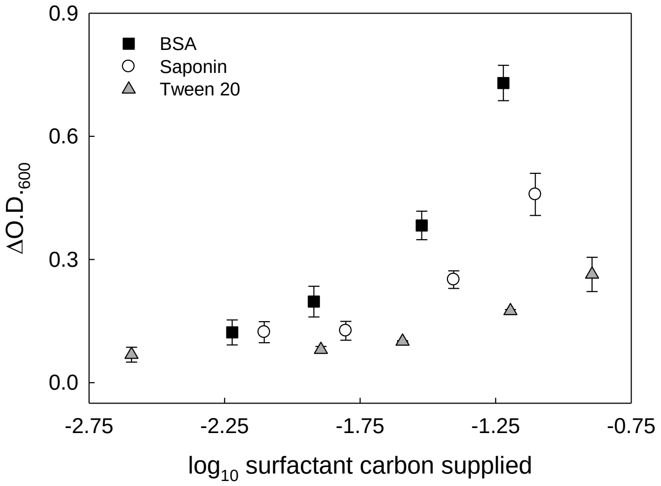


Figure 5.4. Bacterial growth expressed as the increment in optical density at 600 nm after 2.8 days of cultivation (ΔO.D.$_{600}$) in the supernatant of *Scenedesmus obliquus* cultures enriched with BSA, Saponin and Tween 20 at concentrations of 1, 5, 10, 25 and 50 CMC. Surfactant concentration is expressed as the carbon dosage resulting from surfactant addition (logarithmic values, in milligrams). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

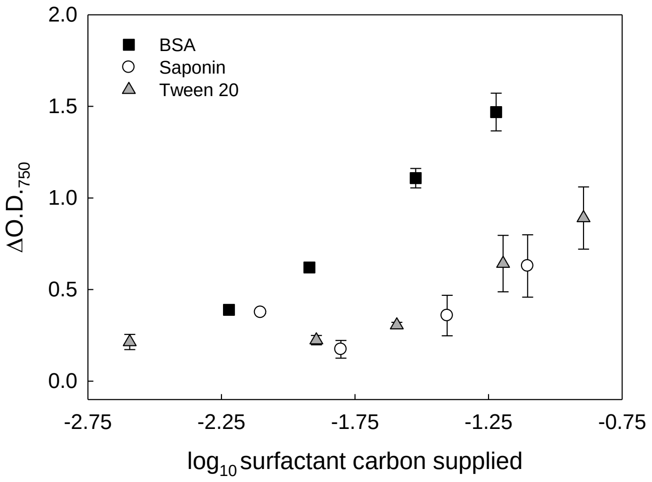


Figure 5.5. *Scenedesmus obliquus* growth expressed as the increment in optical density at 750 nm after 2.8 days of cultivation (ΔO.D.$_{750}$) in cultures enriched with BSA, Saponin and Tween 20 at concentrations of 1, 5, 10, 25 and 50 CMC. Surfactant concentration is expressed as the carbon dosage resulting from surfactant addition (logarithmic values, in milligrams). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

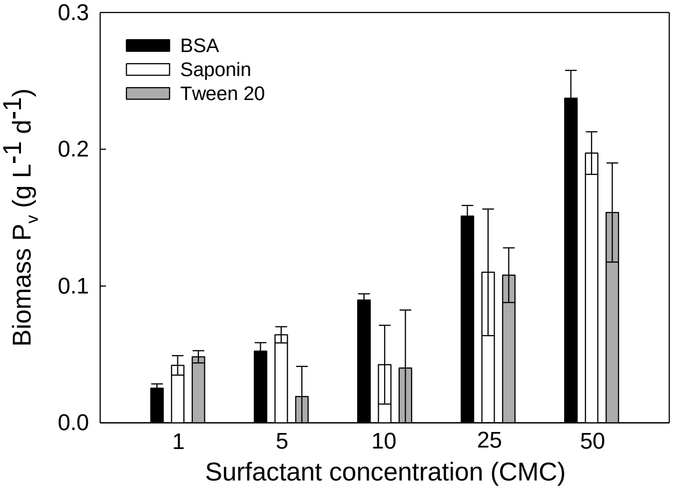


Figure 5.6. Volumetric biomass productivity of *Scenedesmus obliquus* (Biomass P_v), expressed as grams of biomass produced per liter of culture broth and per day after 2.8 days of cultivation in cultures enriched with BSA, Saponin and Tween 20 at concentrations of 1, 5, 10, 25 and 50 CMC. The average values of duplicate cultures are shown, together with their corresponding standard deviations.

Figure 5.4 shows that bacterial growth increased at increasing surfactant concentrations in all cases. This indicates that bacterial growth was stimulated by the three biodegradable surfactants evaluated at concentrations up to 50 CMC. However, same carbon dosages supplied by the different surfactants resulted in different bacterial growth: BSA enhanced bacterial growth to the largest extent while Tween 20 resulted in the lowest bacterial growth. This can be clearly exemplified by the bacterial growth observed in cultures with the largest concentrations (25 and 50 CMC) (**Fig. 5.4**). These differences might be explained by the energy obtained from the degradation of these molecules with respect to the energy used for it. The biodegradation of molecules with high molecular weight and more complex structure might result in less energy production for bacterial growth (Chang et al., 2014). In this sense, there is not apparent correlation between molecular weight/complexity (**Fig. 2.1**, **Table 4.2**) and bacterial growth. However, the differences in the bacterial growth sustained by the different surfactants might be attributed to the degradation of these compounds to a different extent. As aforementioned, BSA molecules have to be hydrolyzed by extracellular enzymes to small peptides that can be transported across the bacterial cell wall (Cadoret et al., 2002; Confer and Logan, 1997; Mosquera-Corral et al., 2003; Wang et al., 2018). This possibly implies that BSA molecules are degraded to a large extent, which would explain the large increase in bacterial growth. Similarly, Saponin has been reported to be completely biodegradable, which in combination with its low molecular weight could compensate its complex structure and be the reason behind the intermediate bacterial growth observed. Lastly, Tween 20 has an intermediate molecular weight (**Table 4.2**) and its structure is not as complex as that of Saponin, however it produced the lowest bacterial growth. This may be due to a partial biodegradation of the molecules, which is in agreement with previous observations (Chang et al., 2014; Yeh et al., 1998).

Microalgal growth was measured as the increment of optical density at 750 nm after 2.8 days of cultivation (**Fig. 5.5**). For the three surfactants, a higher surfactant concentration resulted in increased microalgal growth. The highest algal growth stimulation was found for BSA-added cultures, while Tween 20 and Saponin promoted algal growth to a similar lower extent. The algal growth was approximately 7, 4 and 3-fold higher at 50 CMC of BSA, Saponin and Tween, respectively, than for their corresponding cultures at 1 CMC. The microalgal growth enhancement by the increased surfactant concentrations was visually noticeable (**Fig. 5.2**). It could be explained by the surfactants assimilation by the algal cells or as a consequence of the enhanced bacterial growth, which could result in the release of algal growth-promoting compounds to the broth. The ability of *S. obliquus* to solely degrade these surfactants remains unknown. However, it was previously shown that BSA, which stimulated *S. obliquus* growth to the highest extent, was not consumed by *C. sorokiniana* cells (Janoska et al., 2017) and therefore that hypothesis was disregarded for that specific case. However, the breakdown of the BSA protein by the bacteria might result in peptides or free aminoacids that could possibly be utilized by the microalga. In this sense, the assimilation of peptones (i.e. partially hydrolyzed proteins) and free aminoacids by microalgae has been reported (Flynn and Butler, 1986; Isleten-Hosoglu et al., 2012; Xiong et al., 2008). Likewise, the sugar moiety of Saponin, which is composed of galacturonic acid, glucuronic acid and galactose, might have also been mixotrophically metabolized by *S. obliquus* since some *Scenedesmus* species can grow on more sugars apart from glucose, including galacturonic acid (Tian-Yuan et al, 2014) and galactose (Pancha et al., 2015). On the other hand, Tween 20 might have been preferentially or solely degraded by bacteria. In this sense, Tween 80 was reported to be more intensively utilized by bacteria than by some microalgae (Tuchman et al, 2006). Nevertheless, some *Scenedesmus* strains are able to utilize Tween compounds as carbon source while other microalgal strains are not (Tian-Yuan et al, 2014). Therefore, it is hard to predict if the *S. obliquus* strain used in this study had the ability to utilize Tween 20.

Furthermore, enhanced algal growth could be expected in the cultures where bacteria grew to a higher extent since algal growth can be enhanced by a variety of compounds produced by bacteria, such as CO_2, vitamins, siderophores or growth promoting factors (Fuentes et al., 2016). In this sense, symbiotic relationships have been reported between *S. obliquus* and bacteria (Ferreira et al., 2017) and a maximum of 2.5-fold biomass growth enhancement has been achieved for *S. obliquus* through its co-cultivation with bacteria (Wang et al., 2015b), which could be in agreement with our own findings.

The biomass productivities of *S. obliquus* achieved during the 2.8 days of cultivation in the presence of the surfactants were also calculated and are shown in **Figure 5.6**. As can be seen, increasing BSA, Saponin and Tween 20 concentrations resulted in increased algal biomass productivities. At 50 CMC, biomass productivities were approximately 9.5, 4.5 and 3-fold higher than those at 1 CMC of BSA, Saponin and Tween 20, respectively. As mentioned above, this might be due to the possible metabolization of the surfactants by the microalga or a result of growth-promoting effects of the bacteria on *S. obliquus*.

Furthermore, the biodegradable surfactants showed to affect the bacteria to microalgae ratio differently. The bacteria to microalgae ratio of the cultures at the end of the experiments is shown in **Figure 5.7**. As can be seen, increasing BSA and Saponin concentrations resulted in similar increases of the bacteria to microalgae ratios. However, no effect on this ratio was found in cultures with Tween 20 at any of the concentrations tested, indicating that this surfactant enhanced bacterial and algal growth to a similar extent when added at concentrations up to 50 CMC.

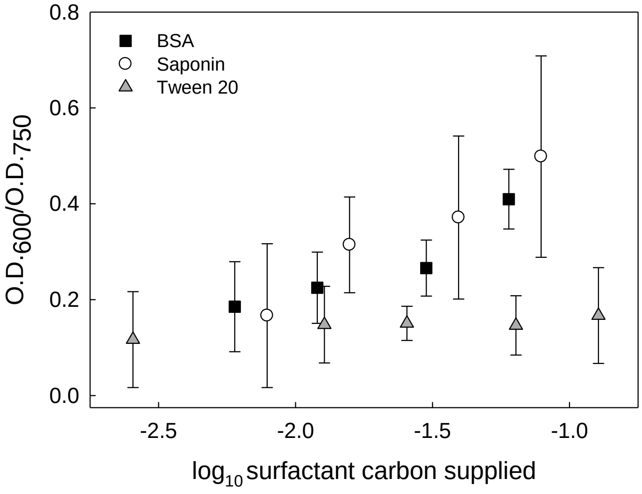


Figure 5.7. Bacteria to microalgae ratio, expressed as the ratio between the optical density at 600 nm and that at 680 nm after 2.8 days of cultivation (O.D.$_{600}$/O.D.$_{680}$) in cultures enriched with BSA, Saponin and Tween 20 at concentrations of 1, 5, 10, 25 and 50 CMC. Surfactant concentration is expressed as the carbon dosage resulting from surfactant addition (logarithmic values, in milligrams). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

5.3.3. Effects of surfactant biodegradation on foaming capacity

The effects of surfactant degradation by *S. obliquus*-associated bacteria on the surfactant foaming capacity were further studied. For that, Saponin was used as example of biodegradable surfactant.

In order to make the biodegradation effects on foamability noticeable, the minimal surfactant concentration that allows to reach the highest foam production speed in our system was experimentally determined. In this sense, the foamability of Saponin solutions at different concentrations was measured as the foam production speed achieved after 30 seconds of foaming (**Fig. 5.8**). According to the results, Saponin was employed at 2 CMC, since that was the minimum surfactant concentration at which the maximum foam production speed (3.4±0.05 mm s^{-1}) was achieved in Luria-Bertani (LB) medium in our system (**Fig. 5.8**). The biodegradability experiment was performed in LB medium because it is a widely used culture medium for the growth of bacteria (Bertani, 1951).

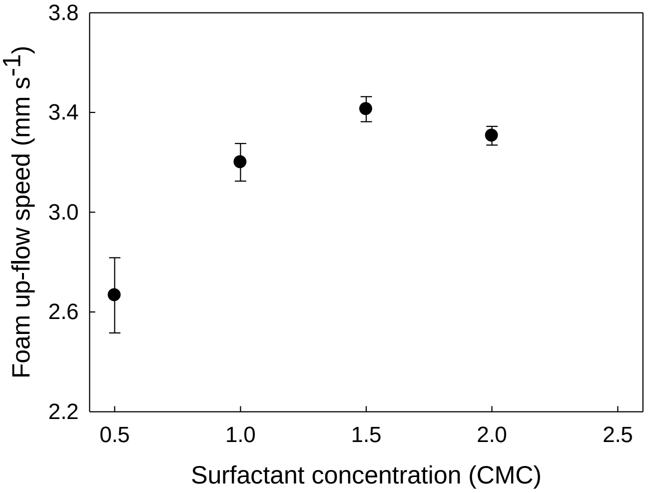


Figure 5.8. Foamability of the surfactant Saponin in 100 mL of LB culture medium expressed in terms of the foam up-flow speed achieved after 30 s of foaming (mm s^{-1}) as a function of the surfactant concentration (CMC). The average values of three experimental replicates are shown, together with their corresponding standard deviations.

The optimal concentration of the bacterial culture medium (LB) was also determined. For that, LB medium diluted 2, 5, 10 and 20-fold was inoculated with *S. obliquus*-associated bacteria and the analysis of optical density at 600 nm allowed to follow the bacterial growth in the different solutions (**Fig. 5.9**). The optimal medium concentration for the biodegradation experiment should ensure that the contained nutrients in the LB diluted media would be depleted before the end of the experiment (in less than 72 hours of cultivation) in order to distinguish when bacterial growth was supported by the use of the surfactant as nutrients and energy source. Hence, inoculated cultures with the addition of the Saponin should be the only ones that might continue growing after the nutrients were depleted from the culture medium.

As can be seen in **Figure 5.9**, only the cultures in 2-fold diluted medium continued growing after 48 hours of cultivation as a result of the still available nutrients in the medium. Besides, it was decided to use 20-fold diluted LB medium to perform the biodegradation experiment since it was the lowest concentrated medium which resulted in bacterial growth cease during the experimental time.

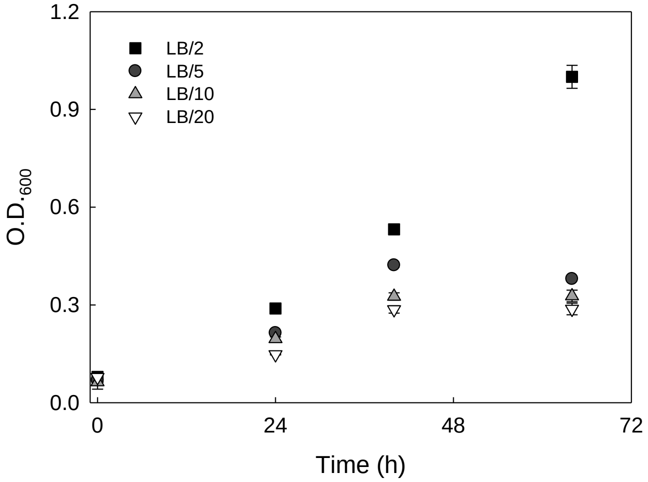


Figure 5.9. Bacterial growth expressed as optical density at 600 nm (O.D.$_{600}$) in LB culture medium diluted 2, 5, 10 and 20-fold. The average values of duplicate cultures are shown, together with their corresponding standard deviations.

Once the optimal surfactant concentration (i.e. 2 CMC) and culture media (i.e. LB/20) were identified, the biodegradability experiment was performed. Bacterial growth (**Fig. 5.10A**) and foamability (**Fig. 5.10B**) were analyzed in non-inoculated LB/20 culture medium, in a Saponin solution prepared in LB/20 culture medium, in bacterial cultures inoculated in LB/20 culture medium, and in bacterial cultures inoculated in a Saponin solution prepared in LB/20 culture medium. Bacterial growth was measured as optical density at 600 nm (**Fig. 5.10A**). As expected, optical density remained constant for non-inoculated cultures during the experimental time, indicating that contamination did not occur in the control culture media (blue dots) or in the Saponin solution (red dots). The bacterial cultures with no Saponin added (green dots) grew during the 2-3 first days, after which they stopped growing due to nutrients depletion. However, the bacterial cultures showed a faster growth when Saponin was present (yellow dots, **Fig. 5.10A**) and bacteria were able to continue growing for 7 days after nutrients from the LB/20 medium were depleted. This indicates that beyond the biodegradation of Saponin, this surfactant supports the growth of *S. obliquus*-associated bacteria. In this sense, it has been stated that microorganisms can either utilize surfactants as substrates to obtain energy and nutrients or co-metabolize surfactants by microbial metabolic reactions (Jurado et al., 2013). Moreover, the fact that the cultures with Saponin grew faster than those without the surfactant, suggest that

S. obliquus-associated bacteria either consumed Saponin and nutrients from LB/20 medium simultaneously, or that Saponin is a more efficient substrate to promote bacterial growth compared to the other nutrients present in the culture media.

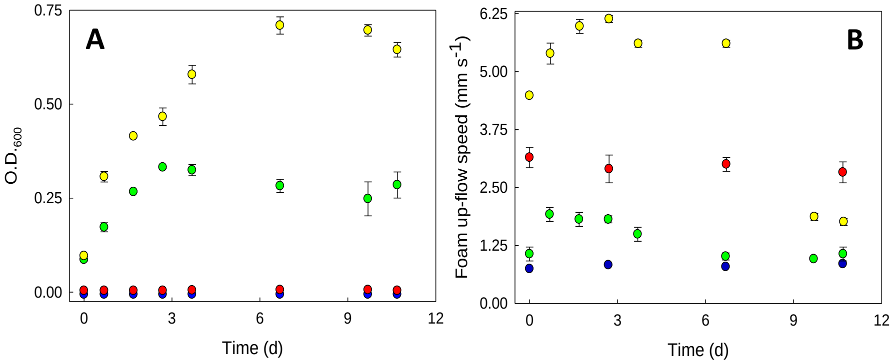


Figure 5.10. (A) Bacterial growth expressed as optical density at 600 nm (O.D.$_{600}$) during the biodegradability experiments with Saponin. (B) Foamability expressed as foam up-flow speed (mm s^{-1}). Culture conditions: LB/20 culture medium without bacteria (blue dots), Saponin solution (red dots), bacterial culture in LB/20 medium (green dots) and bacterial culture in Saponin solution in LB/20 (yellow dots). Saponin was used at a concentration of 2 CMC. The average values of duplicate cultures are shown, together with their corresponding standard deviations.

On the other hand, the foamability of the cultures was measured in terms of the foam up-flow speed achieved in the first 30 seconds of foaming (**Fig. 5.10B**). The foamability of the culture medium (blue dots) remained constant, as well as that of the surfactant solutions (red dots). This indicates that the foamability of Saponin was not affected by the cultivation conditions (i.e. mechanical shaking and incubation at 37 ºC). On the other hand, the foamability of bacterial cultures with no Saponin added (green dots) increased with bacterial growth during the first 24 hours of cultivation, indicating that the presence of bacterial cells promoted the foaming. However, the foamability of these cultures remained constant for the rest of the experiment. Finally, the foamability of the bacterial cultures in the Saponin solution (yellow dots) increased during the first 3 days, which can be explained by the large increase of bacterial cells during that period (**Fig. 5.10A**, yellow dots). However, it was observed that foamability did not proportionally increase with bacterial growth beyond a certain bacterial concentration (green dots). This may explain the foaming capacity of bacterial cultures in Saponin (yellow dots), which remained stable between day 3 and 9 despite the bacterial growth observed.

Lastly, the further bacterial growth of cultures with Saponin supported by the degradation and consumption of this surfactant was confirmed by the drop of the foaming capacity of the cultures with this surfactant, which occurred simultaneously with the cease of growth (**Fig. 5.10A**, yellow dots). It can be suggested that at that time Saponin would have been completely biodegraded and there would be no remaining nutrients to support bacterial growth further. In this sense, the complete biodegradation of this biosurfactant has been already reported (Mølgaard et al., 2000).

5.4 Conclusions

The surfactants BSA, Saponin and Tween 20 had been already proved to be biodegradable in algal-free cultures of the bacteria naturally present in *Scenedesmus obliquus* cultures (Chapter 4). In this Chapter, the surfactants were added to non-axenic mono-algal cultures of *S. obliquus* at concentrations from 1 to 50 CMC. The results showed that BSA, Saponin and Tween 20 enhanced the growth of both the microalga and bacteria naturally present in those cultures in the range of concentrations investigated. The algal biomass productivities were approximately 9.5, 4.5 and 3-fold higher at 50 CMC than those at 1 CMC of BSA, Saponin and Tween 20, respectively. This can be explained by either the assimilation of these surfactants by *S. obliquus* and/or by possible beneficial interactions between the microalga and the bacteria consortia of the cultures, such as the production of algal growth-promoting factors by the bacteria.

When comparing cultures with these surfactants added at the same concentration in terms of carbon dosage, it was observed that BSA promoted algal and bacterial growth to a higher extent that Saponin and Tween 20. On the other hand, Saponin enhanced bacterial growth further than Tween 20, however, these two surfactants promoted algal growth to the same extent when compared in terms of carbon dosage. Overall, increasing BSA and Saponin concentrations in the cultures led to similar increased bacteria-to-algae ratio, while this ratio remained constant in cultures with Tween 20 at concentrations up to 50 CMC.

Besides, it was proved that Saponin can be used as nutrient and energy source by algal-free *S. obliquus*-associated bacteria, which results in a decreased foaming capacity of the cultures.

Overall, despite the supplementation of these biodegradable surfactants in microalgal cultures resulted in microalgal growth enhancement, their use in the LF-PBR would not be suitable due to the consequent foamability losses, as it was proved for Saponin in this Chapter.

Chapter 6

Design, construction and performance of a lab-scale liquid foam-bed photobioreactor prototype

Sections of this Chapter will be submitted as:

Vázquez M, Mogedas B, Cano A, Vílchez C, Cuaresma M. Unveiling the effects of microalgae cultivation in a liquid foam-bed photobioreactor prototype

6.1 Abstract

A lab-scale liquid foam-bed photobioreactor (LF-PBR) was designed in order to have simple, easy-to-build and easy-to-operate foam-bed units to test algae-surfactant interactions in foam-based cultures. The LF-PBR consisted of an air sparger for foam production, two cylinder-shaped vessels for cultivation in foam and liquid collection, a three-dimensional (3D) printed piece for redirecting and breaking the foam, and a silicon tube for liquid recirculation. In addition, two LED (light-emitting diodes) systems were built in order to allow more homogeneous illumination of the LF-PBR and to test different experimental light conditions. In total, four LF-PBR units were built.

The LF-PBR units were tested using Pluronic F68 which was previously determined to be a promising surfactant for microalgae cultivation in foam. Liquid evaporation and surfactant sticking to the system resulted in the need for punctual water and surfactant addition to allow elongated operation periods. Besides, the effect of temperature on the foamability of Pluronic F68 was investigated. The LF-PBR hereby presented allowed uninterrupted foam production and breakage for 5 days using 1L of Pluronic F68 solution, although longer operation times are expected to be achieved by correcting surfactant concentration.

6.2 Introduction

Nowadays, microalgae are produced in liquid-based cultivation systems such as open ponds, tubular photobioreactors and flat panel photobioreactors (Acién et al., 2013, Norsker et al., 2011). However, to make the production of algal products on a large scale economically feasible, limitations such as high operational costs, including high water consumption and energy requirements, should be overcome (Berner et al., 2015). A liquid foam-bed photobioreactor (LF-PBR) has been proposed as an alternative to reduce the costs of microalgae-based production processes (Janoska et al., 2017). This innovative cultivation concept has several potential advantages. First, the pressure drop of a LF-PBR is low due to the reduced liquid content. Thus, the construction costs and the energy spent on gassing could be reduced. Besides, the interfacial area between the liquid containing the microalgal cells and the gas bubbles is much higher in a foam-based culture, resulting in a higher mass transfer of oxygen and carbon dioxide. In this sense, the gas transfer rate has been shown to be one order of magnitude higher than in a liquid bubble column (Janoska et al., 2018a). Also, the long residence time of the CO_2-enriched air bubbles in a LF-PBR leads to a more efficient use of the carbon dioxide. Altogether, higher biomass concentrations can be achieved in a LF-PBR (>20 g L^{-1}) (Janoska et al., 2018a), which may result in a reduction of the harvesting costs by avoiding the first step of biomass pre-concentration, known as primary or bulk harvesting.

Different factors may impact the efficiency of the microalgal cultivation process in a LF-PBR, including those related to the LF-PBR configuration and operational parameters. Besides, the characteristics of the microalgal strain used, the properties of the surfactant employed and its interactions with the algal cells may play a role (Chapter 3 and 4).

Regarding the foam-bed photobioreactor configuration, a flat panel design was used for the first reported LF-PBR (Janoska et al., 2017), since it was hypothesized that its short light path may allow to achieve higher biomass concentrations. Foam production and foam break-up systems were found to be essential for an optimal reactor performance to ensure a sufficient carbon dioxide supply and to avoid oxygen build-up to inhibiting levels (Janoska et al. 2017). Foam can be generated by bubbling a solution which contains a surfactant (Coward et al., 2013). In comparison with other foam generation methods, such as water pressurization, this method has lower energy requirements, few mechanical parts and can easily be scaled up (Laamaen et al., 2016). Besides, foam with higher microalgae content can be generated using a suitable surfactant, since it can interact with the algal cell surfaces (Chen et al., 1998), hence promoting a higher algal partitioning towards the foam phase. On the other hand, foam breakage can be approached by different methods including spontaneous foam destabilization, methods based on physical contact with hydrophobic solid materials, and mechanical methods (e.g. centrifugation, rotating paddles) (Ghildyal et al., 1988; Janoska et al., 2017; Takesono et al., 2002). Mechanical methods are less preferred since they are energy-consuming (Ghildyal et al., 1988). However, very little information is available in the literature on the effect of operational parameters in a foam-bed photobioreactor as research on microalgal cultivation in foam is very scarce yet.

Besides, for a successful microalgae cultivation process in a LF-PBR, a suitable microalga-surfactant combination is needed. The first reported LF-PBR led to the proof of principle of microalgal cultivation in foam (Janoska et al., 2017). The cultivation of *Chlorella sorokiniana* in foam was achieved by using bovine serum albumin (BSA) as foaming stabilizing agent. However, BSA biodegradation and protein denaturation due to foaming limited the foaming capacity of BSA (Chapter 4), and consequently reduced the cultivation time as well. Thus, BSA was determined to be unsuitable for long-term microalgae cultivation in foam and a suitable surfactant for the LF-PBR was stated to be required. The comparison of 10 surfactants in terms of foaming properties, algal partitioning, biodegradability and toxicity led to the selection of Pluronic F68 as a promising surfactant (Chapter 4). Pluronic is the tradename of poloxamers, non-ionic surfactants that consist of triblock copolymers of polyethylene oxide (PEO) and polypropylene oxide (PPO). Pluronic F68 has good foaming properties, it is not biodegradable by microalgae-associated bacteria and it has low toxicity to microalgae. Besides, contrary to BSA, the stability of Pluronic F68 against foaming was shown to be adequate. The foamability of Pluronic F68 was proved stable for twenty cycles of foam generation and deconstruction (Chapter 4). However, the foaming properties of a poloxamer surfactant can be compromised by physicochemical parameters such as pH, ionic strength and temperature (Bonfillon-Colin and Langevin, 1997; Sedev and Exerowa, 1999). Further investigation on how the physicochemical conditions of the LF-PBR can affect Pluronic F68 performance is therefore required.

Furthermore, a relatively low algal partitioning was established as the main drawback of Pluronic F68. In order to overcome this limitation, a new design for the LF-PBR was proposed (Janoska et al., 2018a). It consisted in a small column with a liquid recirculation system that continuously pumped the microalgal suspension from the underlying liquid to the top of the foam column, allowing a more homogeneous algae distribution within the foam-bed column. Long-term cultivation (>500h) of *Chlorella* sp. in Pluronic F68-stabilized foam was achieved in this LF-PBR.

Both reported versions of the LF-PBR allowed microalgae cultivation in foam under very controlled conditions (sterile conditions, control of temperature, pH, foam level) (Janoska et al., 2017, 2018a). Nevertheless, the culture volume (<250 mL in both designs) (Janoska et al., 2017, 2018a) is not enough to perform complete and multiple analysis of the biochemical composition of the biomass along cultivation, which was one of the aims of this Thesis. Thus, in order to study how the surfactant-alga interactions and the physicochemical conditions of cultivation in foam might affect the biochemical composition of the algal biomass, a bigger system was required. Besides, since optimization of the cultivation process is not the aim of this Thesis, a cheap and easy-to-build LF-PBR is also desirable to cheaply and easily obtain many units for lab-scale experiments that may allow to produce biomass cultivated in foam for further analysis.

The aim of this study was, therefore, to design a simple, easy-to-build and easy-to-operate lab-scale liquid foam-bed photobioreactor that could allow mid-term microalgae cultivation in foam, while enabling sufficient culture volume for exhaustive biomass analysis. Besides, illumination systems based on LED (light-emitting diodes) lights were designed, constructed and implemented in order to homogeneously illuminate the LF-PBR. In addition, the LF-PBR prototypes were tested in Pluronic F68-added culture medium and the effect of temperature on the foamability of Pluronic F68 was investigated.

6.3 Results and discussion

6.3.1. Lab-scale liquid foam-bed photobioreactor design

A schematic overview of the LF-PBR setup and its main components is shown in **Figure 6.1**. Foam is produced at the bottom of the LF-PBR by bubbling CO_2-enriched air through a liquid solution which contains a surfactant. The foam-bed column consists of two cylinder-shaped vessels. As foam rises, the first cylinder-shape vessel gets filled with foam, which makes contact with a polymer-based foam-breaker at the top of the LF-PBR. The foam is then redirected to the second cylinder-shaped vessel which has a tilted base. As foam breaks in this vessel, the liquid contained in the foam drains and it is returned to the LF-PBR bottom by gravity through a silicon tube. The gas contained in the foam is released at the top of the LF-PBR through the polymer-based foam-breaker.

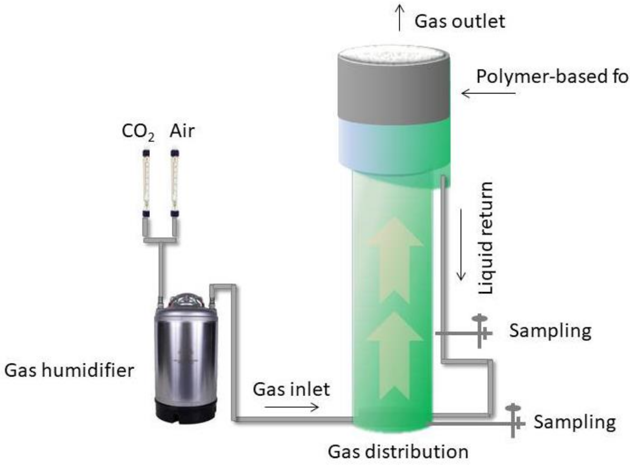


Figure 6.1. Schematic overview of the laboratory scale foam-bed photobioreactor prototype.

Figure 6.2 presents a picture of the LF-PBR (**Fig. 6.2A**) and its dimensions (**Fig. 6.2B**). The LF-PBR had a total height of 0.865 meters and had the capacity to contain approximately 17.12 liters of foam with an illuminated surface area of 0.362 square meters. The dimensions of the cultivation vessels were selected to set up a system that could be operated at lab-scale while containing sufficient liquid culture volume to sample for several biochemical measurements along the cultivation time (i.e. 1L of liquid culture). This volume is approximately 4.5-fold higher than that of the previous reported LF-PBR (Janoska et al., 2017, 2018a).

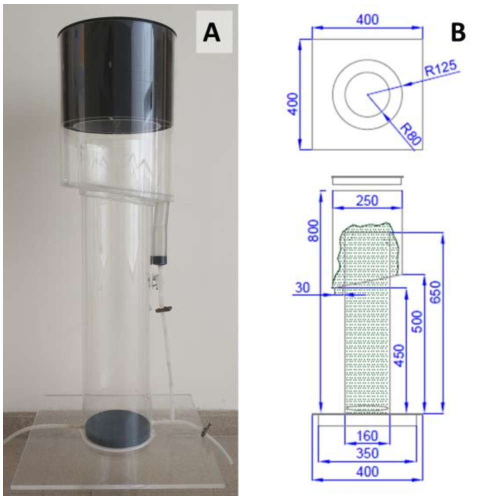


Figure 6.2. (A) Picture of the foam-bed unit with the air-stone diffuser at the bottom part. (B) Top (above) and front (below) scheme views of the foam-bed column. Units are expressed in millimeters.

The foam-bed column consisted in two cylinder-shaped vessels which were made of methacrylate since this material is transparent, inexpensive and it is easy to work with. An aquarium air-stone diffuser was placed at the bottom of the LF-PBR occupying the whole bottom surface of the first vessel in order to produce a homogeneous foam column and prevent biomass sedimentation at the bottom of the reactor. The gas (i.e. CO_2-enriched air for microalgae cultivation) was supplied via two rotameters (for air and CO_2), which allowed to adapt the gas flow and the final CO_2 concentration in the gas mixture. The CO_2-enriched air (2.71% v/v) was pumped through a 2L pressurized water bottle in order to be humidified and minimize evaporation before entering the LF-PBR through the air diffuser. The base of the second cylinder-shaped vessel was tilted in order to allow the liquid obtained once foam breaks to drain by gravity to the recirculation tube. The recirculation tube was made of silicone, which showed to help destabilize the foam in case some foam entered the tube along with the liquid. Silicone-based materials have been proposed to act as antifoam (i.e. preventing foam generation) or defoamers (i.e. promoting foam deconstruction) (Jha et al., 2000; Pouchelon and Araud, 1993; Pugh, 1996). A sampling port was placed in this tube in order to collect samples of the liquid contained in the foam. Another sampling port was placed at the bottom of the LF-PBR, right above the diffuser, to allow sampling directly from the liquid phase.

The fact that the liquid recirculates without extra pumping and that the foam is not broken by any mechanical method, but spontaneously, would make the gassing and illumination system the only energy-consuming processes in this prototype. In general terms, the gassing costs are expected to be much lower than those of a liquid photobioreactor due to the long residence time of the bubbles and the more efficient use of the CO_2 (Janoska et al., 2018a). This, along with the low cost materials used to build the prototype, might make the designed LF-PBR an economic microalgae cultivation system for laboratory research.

In the absence of the polymer-based foam breaker, the arising foam column showed to continue arising instead of fall into the second vessel, resulting in suboptimal performance of the LF-PBR. Thus, a polymer-based foam breaker was designed (**Fig. 6.3A**) in order to redirect the foam towards the second vessel and to allow the release of the gas contained in the foam, which at that moment should be ideally depleted in CO_2 and enriched in O_2. The gas release is indicated by the dashed green arrows in **Figure 6.3C**. **Figure 6.3B** shows the top and bottom view of the piece, which was built by 3D-printing and made of polylactic acid (PLA). This thermoplastic is considered biodegradable and biocompatible (Lasprilla et al., 2012) and it is widely used in 3D-printing (Wang et al., 2017). During normal operation, the polymer-based foam breaker is placed in the second vessel (**Fig. 6.2A**). When the arising foam reaches the foam-breaker, it is redirected into the second vessel as indicated by the solid green arrows in **Figure 6.3C**. The contact of foam and the polymer-based foam breaker promoted foam deconstruction, probably due to the properties of the selected material, such as hydrophobicity or roughness (Kadoi and Nakae, 2011).

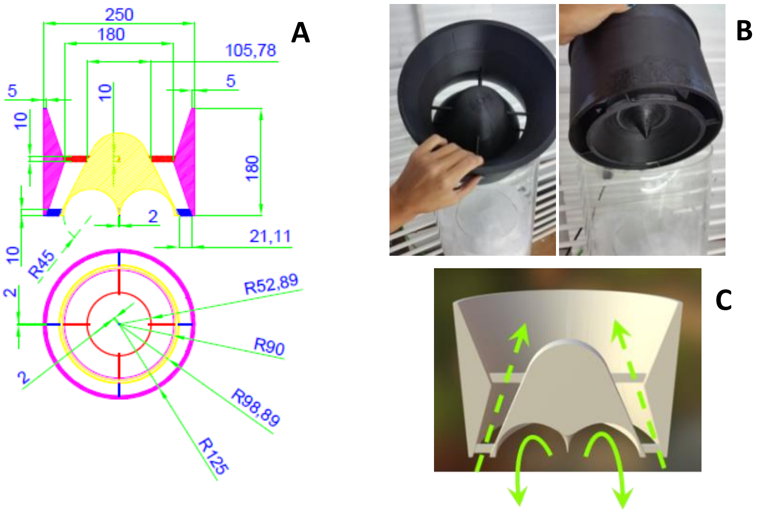


Figure 6.3. (A) Front and bottom view of the polymer-based foam breaker. Measurements are expressed in millimeters. (B) Pictures showing the top (left) and bottom (right) views of the polymer-based foam breaker. (C) Full sectional front view of the polymer-based foam breaker. Solid arrows represent the flows of the foam which is redirected to the second vessel of the foam-bed column; dashed arrows indicate the gas exchange for oxygen release.

Two different systems were used to illuminate the LF-PBRs: a panel of fluorescent lamps (**Fig. 6.4A**) and a cylindrical LED-system (**Fig. 6.4B**). The panel system was formed by 10 warm white fluorescent lamps (Phillips Master 54W/830) placed in horizontal position. This system allowed the illumination of the LF-PBR from one side only. Hence, a LED-system was designed in order to provide more homogeneous illumination to the LF-PBR and with higher light intensities. The LED-system consisted of sixteen LED bands placed in vertical position, resulting in a cylindrical framework. Each LED band consisted of twelve 5000K LED lights: nine 15W LED lights illuminating the lower vessel, and three 9W LED lights for the illumination of the upper vessel. Less powerful LED lights were used for the upper part of the system in order to compensate the shorter distance of the upper vessel to the lights, hence to illuminate the foam more homogeneously at the surface. The emission spectra of the fluorescent lamps and the LED lights are shown in **Figure 6.4C** and **6.4D**, respectively.

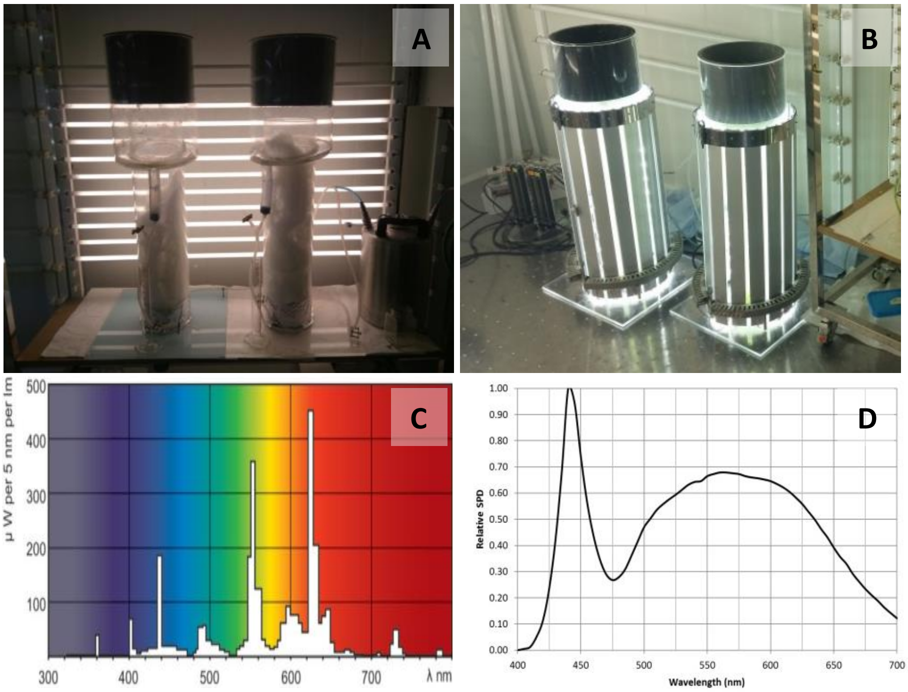


Figure 6.4. Liquid foam-bed photobioreactors illuminated by: (A) panel of fluorescent lamps and (B) LED-systems. Emission spectra of the lamps employed to illuminate the liquid foam-bed photobioreactors: (C) fluorescent lamps of the panel system, expressed as µW nm^{-1} lm^{-1}; and (D) LED lights used for the cylindrical LED-system, expressed as relative spectral power distribution (SPD).

In order to characterize the illumination provided to the LF-PBR by the two illumination systems, the light intensity was measured inside the LF-PBR units at four different heights (**Fig. 6.5**). At each height, the measurement was done four times around the perimeter of each LF-PBR unit. The radiometer probe was placed just behind the LF-PBR walls, as indicated in **Figure 6.5**. The light intensity of the LED-systems was regulated by light dimmer switches, which were set at three different positions to characterize a minimum, an intermediate, and a maximum irradiance that can be provided by this system. On the other hand, the light emission spectrum provided by the panel of fluorescent lights was characterized at its maximum intensity.

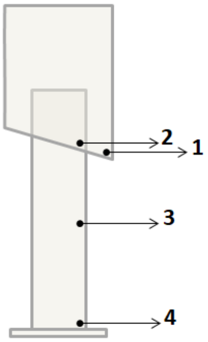


Figure 6.5. Scheme of liquid foam-bed photobioreactor showing the four points where the irradiance was measured.

The light intensities measured for both illumination systems are shown in **Table 6.1**. The light panel provided an average light intensity of 183±23 µmol photons m^{-2} s^{-1}. The light intensity of the LED-systems could be set at average light intensities ranging from 157±31 to 431±75 µmol photons m^{-2} s^{-1}.

Table 6.1. Light intensity provided by the panel of fluorescent lights and the LED-system. For the latter, the measurements were done at three light intensities (minimum, intermediate, maximum). The light intensity was measured four times around the LF-PBR at each measuring point (see Figure 6.5). The measurements were done in two LF-PBR units. The average intensity is the mean of the intensities at the four measuring points, with the corresponding standard deviations.

	Light intensity (µmol photons m^{-2} s^{-1})			
Measurement point	**Fluorescent lamps panel**	**LED-system**		
		Minimum	**Intermediate**	**Maximum**
1	204.0 ± 8.5	145.0 ± 10.6	243.3 ± 2.5	432.1 ± 39.4
2	185.1 ± 6.9	127.0 ± 4.2	179.8 ± 20.9	338.9 ± 28.5
3	196.7 ± 2.1	203.5 ± 9.2	303.3 ± 2.5	531.1 ± 8.7
4	148.2 ± 5.7	155.8 ± 10.3	231.5 ± 6.4	423.2 ± 0.0
Average	183.5 ± 23.4	157.8 ± 31.0	239.4 ± 47.7	431.3 ± 75.2

6.3.2. Lab-scale liquid foam-bed photobioreactor performance

Previous results (Chapter 3 and 4; Janoska et al., 2018a) showed the potential of *Chlorella sorokiniana* to be cultivated in foams stabilized by Pluronic F68. Thus, the performance of the LF-PBR was tested using a Pluronic F68 solution prepared in

M-8, which is the algal culture medium used for the cultivation of *C. sorokiniana*. Since the LF-PBR lacks a temperature control system, the different units were placed in a culture room under controlled temperature (25 °C). The initial liquid volume, surfactant concentration and gas flow rate were adjusted in order to: (i) completely fill the LF-PBR of foam, (ii) keep the liquid phase at the bottom of the reactor at a minimum volume, and (iii) produce foam with an intermediate stability to allow its breakage at the top (i.e. balanced liquid recirculation and aeration flow rates). The operational conditions used are enlisted in **Table 6.2**. The initial liquid volume was one liter, since it allowed to fill the LF-PBR of foam (i.e. approximately 17 liters of foam). For an optimal foam stability, Pluronic F68 was used at 12.5 CMC. Next, a range of gas flow rates (from 260 to 4550 mL min^{-1}) was tested. The optimal gas flow rate was determined as 3000 mL min^{-1}, since it allowed the LF-PBR to operate continuously with minimum remaining liquid at the bottom. Lower gas flow rates led to the formation of drier foams with most of the liquid remaining at the bottom. On the contrary, at higher gas flow rates all the liquid was converted into foam and the CO_2-enriched air entering at the base of the foam column produced the foam to overflow the unit. The lower 8 cm of the LF-PBR were covered with aluminium foil (**Fig. 6.4A**) in order to avoid illumination of the liquid above the diffuser when cultivating microalgae in foam.

Table 6.2. Operational conditions of the liquid foam-bed photobioreactor prototypes.

Parameter	Foam-based
Culture medium	M-8
Surfactant and concentration	Pluronic F68, 12.5 CMC
Gas flow rate	3000 mL min^{-1}
Superficial gas flow rate	$2.5\ 10^{3}$ m s^{-1}
CO_2 enrichment	2.71% (v/v)
Illumination system	Fluorescent lamps panel

Two LF-PBR units were run in parallel for 5 days. Samples were taken daily and observed under optical microscopy in order to check for possible contamination, since the LF-PBR are open systems and the LF-PBR and surfactant solutions were not sterilized. Besides, liquid evaporation was measured and corrected with distilled water daily. Liquid evaporation rate was in average 167±74 mL d^{-1} for each LF-PBR, which accounts for about 17% of the total LF-PBR volume.

In order to investigate the fate and stability of Pluronic F68 in the LF-PBR, its concentration was measured daily in the total liquid and in the liquid released from the foam (**Fig. 6.6**). The surfactant concentration in the total liquid refers to the surfactant concentration in the whole liquid volume contained in the LF-PBR, since it was measured after letting all the foam collapse, and once the liquid evaporation was corrected. On the other hand, the surfactant concentration in the liquid released from the foam was measured in samples taken from the recirculation tube while the LF-PBR was operating, hence before correcting the evaporation. Thus, the latter was recalculated considering the volume of water added afterwards in order not to overestimate the values measured.

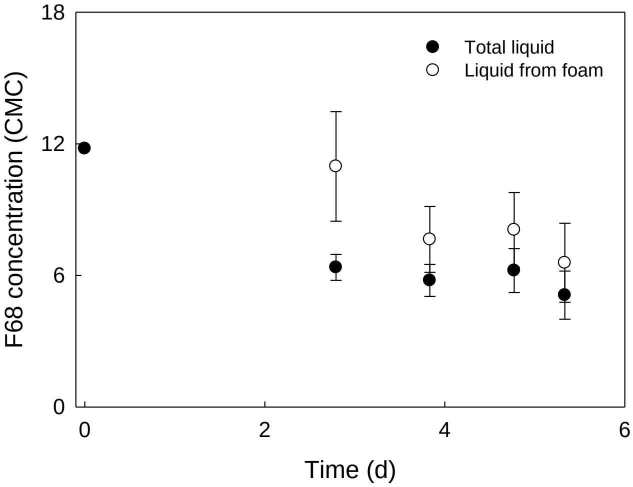


Figure 6.6. Pluronic F68 concentration in the total liquid and in the liquid released from the foam. The average values of samples in triplicate from two liquid foam-bed photobioreactors are shown, together with their corresponding standard deviations.

Pluronic F68 was quantified using a method that is based on its chemical derivatization and posterior colorimetric detection adapted from Chung and co-workers (2011) (see Materials and Methods, **Section 2.6.1**). As can be seen in **Figure 6.6**, the surfactant concentration in the total liquid and in the foam decreased over time, being higher in the foam phase. When the LF-PBR was in operation, foam was formed and the liquid contained in it had a higher surfactant concentration (**Fig. 6.6**) which simultaneously decreased in the liquid phase. In accordance with our results, it is known that foaming a surfactant solution results in a decrease of the surfactant concentration in the liquid phase (Boos et al., 2012). However, the remaining surfactant concentration in the liquid phase showed to be enough to sustain foam generation during the experimental time. Moreover, the decrease in surfactant concentration over time was related to the surfactant sticking to the system. This was particularly evident in the polymer-based foam breaker, where residues of the surfactant accumulated during the experimental time (**Fig. 6.7**). Similar observation of Pluronic F68 decrease in the LF-PBR were made by Janoska and co-workers (2018a), although the reasons behind were not determined in their work.

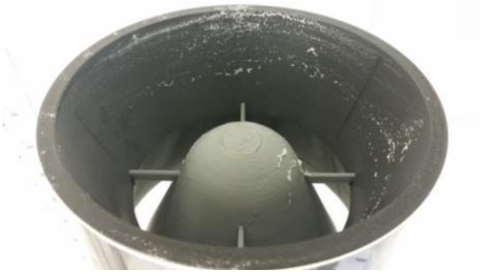


Figure 6.7. Picture of the polymer-based foam breaker showing residues of Pluronic F68 on it.

6.3.3 Effect of temperature on Pluronic F68 foamability

When the LF-PBR units were illuminated with the LED-systems, the foamability of the surfactant solution decreased drastically overnight. As a consequence of the moderate irradiances supplied, these systems produced an increase in the solution temperature. The effect of temperature on the foamability of the surfactant Pluronic F68 was then investigated by incubating surfactant solutions in M-8 culture medium at 18 and 37 ºC (see Materials and Methods, **Section 2.4.7**). The former was chosen as reference temperature, since it was similar to the temperature at which foaming properties were studied (Chapter 4). The thermosensivity of Pluronic F68 was studied at 37 ºC since it was the temperature achieved in the LF-PBR units (liquid phase) when these were illuminated by the LED-systems. The surfactant solutions were incubated for a total of 67 hours. Replicates were used to measure the foamability of the Pluronic F68 solutions in the LF-PBR at different incubation times. The obtained results are presented in **Figure 6.8** and expressed as the foam-up flow speed achieved until the foam reached the top of the LF-PBR (i.e. 60 cm of height).

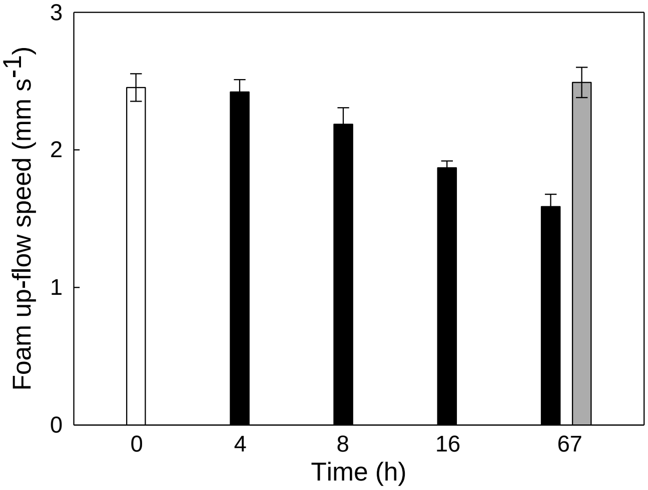


Figure 6.8. Foamability of Pluronic F68 solutions prepared at 12.5 CMC in M-8 culture medium, expressed as the foam up-flow speed (mm s^{-1}) achieved after 4, 8, 16 and 67 hours of incubation at 18 ºC (grey bar) and at 37 ºC (black bars). The initial foamability of the surfactant solutions is also presented (white bar). The average values of three experimental replicates are shown, together with their corresponding standard deviations.

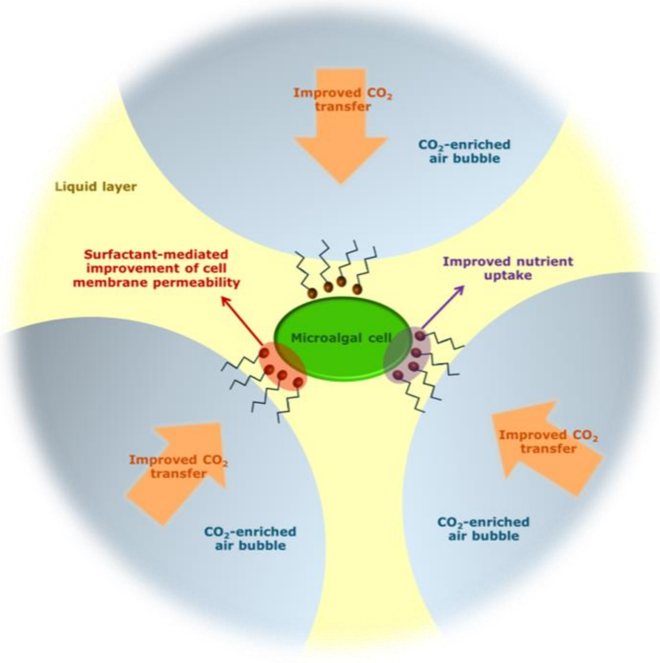
As shown, the foamability of the Pluronic F68 solutions decreased over time when incubated at 37 °C, while it remained constant when incubated at 18 °C. A 35% reduction in foamability was found after incubation at 37 °C for 67 hours. This indicates a negative impact of temperature on the foamability of the surfactant Pluronic F68. Our results are in agreement with the decrease of stability observed for Pluronic F68-stabilized foams during the cultivation of *Chlorella* at 37 °C (Janoska et al., 2018). The degradation of Pluronic F68 has been reported to take place at 40 °C, while its stability at 25 °C was found satisfactory (Erlandsson, 2002). This study indicated that the degradation of Pluronic occurs due to autooxidation reactions that lead to chain cleavage (preferentially C-C but also C-O) at the PPO and PEO parts, the PPO part being more easily degraded. The degradation of Pluronic F68 leads to the production of acids (acetic and formic) and aldehydes (acetaldehyde and formaldehyde), resulting also in a pH decrease. Despite the pH was not measured in the present experiment, spectrophotometric quantification of Pluronic F68 concentration did not show any decrease along the incubation time period at 37 °C (data not shown). This might be due to our short experimental time compared to the 6-month experiment performed by Erlandsson (2002). Next to the improbable chemical degradation of Pluronic F68 in our short assay, the surfactant properties, such as foamability, depend on the adsorption of the molecules into the liquid/gas interphase among other factors. It has been suggested that Pluronic molecules fold at the liquid-gas interphase, with the PPO groups looping away from the aqueous phase and the PEO groups extending into the aqueous phase (Alexandridis et al., 1994). It is known that increased temperatures result in reduced effectiveness of this adsorption process (Rosen, 2004). Another factor that influences foaming properties is the formation of micelles, which contributes to the stabilization of the foam and increases its liquid content (Beneventi et al., 2003). In this sense, previous studies have reported that Pluronic surfactants exhibit temperature-dependent micellization characteristics in aqueous solutions (Bae et al., 2006). At higher temperatures, the weakened interaction between Pluronic and water molecules results in water being gradually excluded from the micelles (Nivaggioli et al., 1995; Su et al., 2002). Besides, the foam stability might have been reduced at higher temperatures due to increased foam disproportionation. Disproportionation refers to the migration of gas between bubbles leading to coarsening of the foam. In this sense, it has been reported that foam disproportionation increases with the temperature in Pluronic F68-stabilized foams (Gandolfo and Rosano, 1997). All the aforementioned studies are in line with the observations shown in this study, and the mechanisms discussed may be behind the loss of foamability hereby observed for Pluronic F68 at 37 °C.

6.4 Conclusions

A liquid foam bed photobioreactor prototype (LF-PBR) was designed, built and set up in order to cultivate microalgae in foam at lab scale. The LF-PBR basically consisted of two cylinder-shaped methacrylate vessels, a porous stone used as air diffuser and a 3D-printed top piece as foam-breaker. The performance was tested using Pluronic F68 solutions. Under the optimal gas flow rate, one liter of Pluronic F68 solution (12.5 CMC) allowed uninterrupted foam production and breakage for 5 days. However, water evaporation had to be corrected daily. Besides, the surfactant showed to stick to the system resulting in a decreasing surfactant concentration over time. In addition, LED-systems were constructed in order to provide the LF-PBR with a more intense and uniform illumination. Apart from the illumination, the gassing system was the only energy source used for the operation of the LF-PBR. Increases in the solution temperature showed to decrease Pluronic F68 foamability. However, LF-PBR might operate for longer periods of time by correcting both the liquid evaporation and the decrease of surfactant concentration. Overall, a laboratory photobioreactor prototype was designed which enabled the production of a stable foam-bed in the cultivation vessel based on the continuous production of foam and its breakage at the top piece. This system allows testing production of microalgae in surfactant-stabilized foam.

Chapter 7

Unveiling the surfactant effect on the composition of microalgae cultivated in a liquid foam-bed photobioreactor



Sections of this Chapter will be submitted as:

Vázquez M, Mogedas B, Cano A, Vílchez C, Cuaresma M. Unveiling the effects of microalgae cultivation in a liquid foam-bed photobioreactor prototype

Vázquez M, Montero Z, Gommers E, Platvoet D, Vílchez C, Cuaresma M. Biomass quality of microalgae cultivated in a liquid foam-bed photobioreactor prototype

7.1 Abstract

For a successful microalgae cultivation process in a liquid foam-bed photobioreactor, a suitable surfactant-alga combination is needed. In this sense, previous results shown in this Thesis have stated the potential of *Chlorella sorokiniana* to be cultivated in foams stabilized by the surfactant Pluronic F68.

Microalgae cultivation in surfactant-stabilized foams might have an impact on the quality of the biomass and its posterior applications due to possible interactions of the cells with the surfactant and/or the particular characteristics imposed by the "foam environment". In this work the effects of Pluronic F68 on liquid and foam-based microalgal cultures were investigated under different scenarios, including nitrogen starvation and/or the use of saturating light. The main results regarding microalgae viability, cell integrity and the biochemical composition changes produced in the cells (i.e. content and profile of fatty acids, proteins, carbohydrates and pigments –lutein- content) are shown.

7.2 Introduction

Microalgae cultivation in foam has been proposed as a promising alternative to conventional cultivation systems which may lead to cost reduction of microalgae-based production processes (Janoska et al., 2017). To accomplish foam-based microalgae cultivation, three liquid foam-bed photobioreactors (LF-PBR) have been developed so far (Janoska et al., 2017, 2018a; Chapter 6). The operation of all the reported LF-PBR is based on the bubbling of a liquid algal suspension which contains a surfactant, resulting in the production of surfactant-stabilized algal-enriched foam which is illuminated to promote microalgal growth. Algal biomass is the product obtained in the cultivation process in foam. The quality of the biomass obtained in a microalgae production process is a key aspect that must be carefully addressed in order to ensure its subsequent use in an array of applications. The potential applications and commercial value of a specific microalga species depend –among other factors- on the composition of the produced algal biomass in valuable compounds, namely fatty acids, carotenoids, carbohydrates, proteins and other molecules (Enzing et al., 2014). The process for the production of algal biomass should be such that the biomass can be obtained in a systematic way and attaining the same composition in target compounds on every harvesting cycle. Due to the novelty of the foam-bed photobioreactor concept, little knowledge is available on systematic cultivation of microalgae in surfactant-stabilized foam, and the dynamics of the biochemical composition of the foam-based cultivated biomass have not been studied yet.

In a LF-PBR, the surfactant-stabilized algal-enriched foam is composed of gas bubbles surrounded by a thin layer of an aqueous cell suspension which contains surfactant molecules with both hydrophobic and hydrophilic domains. The algal cells grow in these thin liquid layers that surround the gas bubbles and are in contact with the surfactant molecules. The potential interactions between surfactant and algal cells might induce changes in one or several biochemical features, including membrane permeability, cell metabolism and major biochemical profile of the cells. The aforementioned surfactant-algal cells interactions may for instance impact the primary cell metabolism, particularly the uptake of essential nutrients (Singh et al., 2016; Taoka et al., 2011) resulting in changed biochemical composition. A recent study (Esakkimuthu et al., 2019) showed enhanced growth of *Tetradesmus obliquus* when supplementing the culture with several polysorbitol surfactants (tradename Tween) due to increased membrane permeability but also due to the usage of these surfactants as carbon source. Besides, Esakkimuthu and co-workers (2019) found that a suitable concentration of surfactant led to higher lipid contents in the biomass. According to the stated above, the possibility exists that long-term interaction of microalgal cells with surfactant molecules during the cultivation in a foam-bed reactor may result in changes in the growth pattern and the major biochemical composition of the algal cells, and thus in biomass of a different quality. Consequently, cultivation in foam could be investigated as a tool to modify the major biochemical composition of the microalgal cells, i.e. content and profile of fatty acids, proteins, carbohydrates and pigments.

Besides, the selection of a suitable alga-surfactant combination has been deemed necessary for a proper operation of the LF-PBR. In this sense, the cultivation of *Chlorella sorokiniana* in Pluronic F68-stabilized foams was found to be promising (Chapter 4), and foam-based cultivation in a LF-PBR was proved successful using this alga-surfactant combination (Janoska et al., 2018a). Since the effects of surfactants on microalgae depend on the particular surfactant and the microalgal strain (Chapter 4), how the surfactant Pluronic F68 may impact *C. sorokiniana* growth and biomass quality should be particularly studied. Pluronics are bioactive surfactants able to interact with biological membranes (Doğan et al., 2012). In particular, Pluronic F68 has been reported to show mostly positive effects on microbial cultures (Ntwampe et al., 2010). Pluronic F68 has been widely used as a protective agent and growth promoting additive in different eukaryotic cell cultures, including mammalian (Bentley et al., 1989; Clincke, 2011), insect (Murhammer and Goocher, 1988; Palomares et al., 2000), plant (Khatun et al., 1993; Kumar et al., 1992) and microalgae cells (Gallardo-Rodríguez et al., 2011; Sobczuk et al., 2006; Zhao et al., 2016). Pluronic F68 was also shown to prolong the survival of the protozoa *Tetrahymena* when subjected to nutrient starvation among other physical and chemical stress conditions (Hellung-Larsen et al., 2000).

However, there is limited information on the effect of Pluronic F68 on algal cultures. The lack of toxic effects of Pluronic F68 on *C. sorokiniana* when added to liquid cultures at concentrations up to 50 CMC has already been proved (Chapter 4). Besides the studies on the toxicity of Pluronic F68, there are few other studies focused on the shear protectant effect of this surfactant (Gallardo-Rodríguez et al., 2011; Sobczuk et al 2006; Zhao et al., 2016). For instance, it has been reported that Pluronic F68 protected *Porphyridium cruentum* cells from the damage caused by the rupture of the gas bubbles at the liquid surface (Sobczuk et al., 2006). Similarly, Camacho and co-workers (2007) demonstrated the shear protectant effects of Pluronic F68 in aerated cultures of the shear-sensitive dinoflagellate *Protoceratium reticulatum*, which are very sensitive to bubbling. However, the precise shear-protectant mechanism in algal cultures remains undetermined. Only Zhao and co-workers (2016) studied this in terms of microalgal cell membrane integrity, and it was concluded that the addition of Pluronic F68 improved the cell membrane integrity of *Chlamydomonas reinhardtii* when exposed to a pyrolitic substrate that produces membrane damage, resulting in increased cell growth. However, increased uptake of fluorescein diacetate by yeast cells was observed due to Pluronic F68 addition (King et al., 1991), and it was suggested that it may have been caused by the formation of membrane pores.

On the other hand, the physicochemical conditions in the foam (i.e light and CO_2 disponibility) differ from those in liquid cultures. In particular, the CO_2 transfer may be increased, thus enhanced growth would be expected. How the "foam environment" may impact the biochemical composition of the foam-based algal biomass remains to be investigated.

Altogether, the particular conditions faced by the algal cells growing in surfactant-stabilized foam (i.e. surfactant interactions with the algal cells and physicochemical conditions of the foam) constitute a distinctive scenario that may have an effect on the algal growth as well as on the biomass composition. This study is aimed at unveiling the changes in growth and the major biochemical composition of *C. sorokiniana* in both Pluronic F68-added liquid cultures and Pluronic F68-stabilized foam-based cultures. For that, conventional lab-scale liquid cultivation systems (i.e. Erlenmeyer flasks) and the LF-PBR designed in Chapter 6 were used. Besides, this study includes several stress scenarios including nutrient starvation and saturating light intensities in order to get a first sight on how foam-based microalgal cultures would perform under these stress conditions.

7.3 Results and discussion

7.3.1 Interactions between Pluronic F68 and algal cells. Effects on algal cell surface hydrophobicity

As a first approach to study the interactions between the surfactant and the microalgal cells, the effect of the surfactant Pluronic F68 on the cell surface hydrophobicity of four microalgal strains was investigated (**Table 7.1**). This parameter has been closely related to the efficiency of microalgal flotation (Garg et al., 2012) and may therefore play a role in the performance of microalgal cultures in surfactant-stabilized foam. Cell surface hydrophobicity was tested using a modified adherence-to-hydrocarbon method (see Materials and Methods, **Section 2.4.3**). The cell surface hydrophobicities without surfactant and with the addition of bovine serum albumin (BSA) were used as a reference, since BSA has been previously employed as a model surfactant in several related studies (Chapter 3; Janoska et al., 2017; Vázquez et al., 2018).

Table 7.1. Microalgal cell hydrophobicity (%) of samples obtained from cultures growing in linear phase, without the addition of any surfactant, in the presence of BSA at 0.1 g L^{-1} and with Pluronic F68 at 1 CMC. Microalgal strains: *Botryococcus braunii* CCALA-778 (B. b. C), *Chlorella sorokiniana* (C. so), *Nannochloropsis gaditana* (N. ga) and *Scenedesmus obliquus* (S. ob). The average values of three experimental replicates are shown, together with their corresponding standard deviations.

	B. b. C	**C. so**	**N. ga**	**S. ob**	**Reference**
Natural hydrophobicity (%)	0.93 ± 2.50	27.99 ± 6.98	14.81 ± 1.12	65.25 ± 5.67	Vázquez et al., 2018; Chapter 3
BSA-added hydrophobicity (%)	48.52 ± 6.74	30.12 ± 9.24	5.21 ± 1.10	57.43 ± 1.58	Vázquez et al., 2018; Chapter 3
Pluronic F68-added hydrophobicity (%)	0.20 ± 0.16	18.44 ± 0.03	1.22 ± 0.01	0.67 ± 0.13	This chapter

It can be inferred from the results that microalgal hydrophobicities were lower with the addition of Pluronic F68 than with BSA, and lower than the surfactant-free hydrophobicities (**Table 7.1**). The low cell surface hydrophobicity obtained with Pluronic F68 might have contributed to the relatively low algal partitioning towards the foam of *C. sorokiniana* and *S. obliquus* previously described (Chapter

4, **Fig. 4.3**). Nevertheless, previous results showed that the microalgal partitioning towards the foam phase must be also influenced by other parameters (Chapter 3).

7.3.2 Pluronic F68 effects on liquid cultures

The interaction between Pluronic F68 molecules and microalgal cells was further investigated in microalgal liquid cultures. In order to evaluate the strength of these interactions and the fate of the surfactant in the cultures (i.e. to determine whether the surfactant molecules stick to the algal cells or remains in solution), the surfactant concentration was measured several times during cultivation. The experiments were performed with *C. sorokiniana* because of the large potential of this microalga to be cultivated in foam (Chapter 3). Besides, this strain showed the highest Pluronic F68-added cell surface hydrophobicity (**Table 7.1**), which could be beneficial for their partitioning towards the foam phase.

Pluronic F68 was added to cultures under no nutrient starvation and to nitrogen-starved (N-starved) cultures in order to determine possible differences in the interaction with non-stressed cells and cells growing under nutrient deprivation. Pluronic F68 was added at a concentration of 12.5 CMC since this concentration was determined to be optimal for the liquid foam-bed photobioreactor operation (Chapter 5). The evolution of the surfactant concentration measured in the liquid broth during cultivation is shown in **Figure 7.1**.

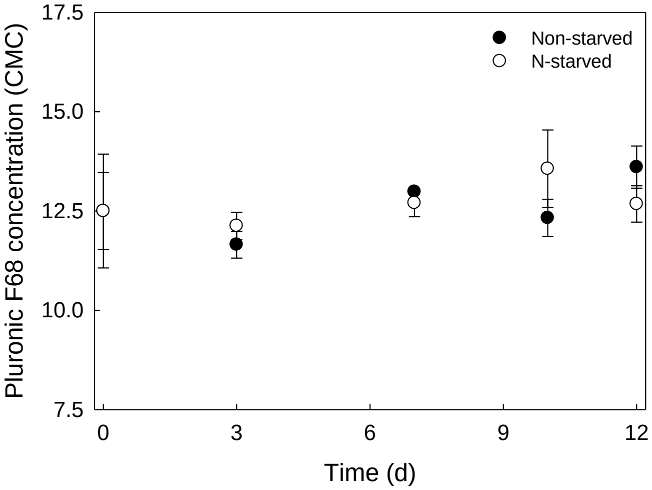


Figure 7.1. Evolution of the concentration of Pluronic F68 in non-starved and N-starved liquid cultures of *Chlorella sorokiniana*. The average values of samples analyzed at least in triplicate are shown, together with their corresponding standard deviations.

The surfactant concentration in the cultures remained constant during the experimental time (up to 12 days). This indicates that there was no assimilation of the surfactant by the cells or strong physical attachment of the Pluronic molecules to the algal cell surfaces of *C. sorokiniana* (**Fig. 7.1**). Irreversible interactions of the surfactant and the algal cells would have resulted in a decrease of the Pluronic concentration in the cultures, since the molecules would have been lost together with the biomass at the beginning of the Pluronic quantification method when the culture sample is centrifuged after the addition of trichloroacetic acid (TCA) and methanol (see Materials and Methods, **Section 2.6.1**). However, the possible interactions between the surfactant and the algal cells might as well have been reverted in the abovementioned step due to the addition of TCA and methanol. Therefore, our results suggest that the interactions between Pluronic F68 and *C. sorokiniana* cells in liquid cultures, if existing, might have a weak and reversible character.

Pluronic F68 has been used as a protectant against physical and chemical stress, as a growth-promoting factor and to prevent cell adhesion in cultures of a variety of cells including bacteria, yeast, protozoa, algae, plant and animal cells (Au et al., 2011; Lowe et al., 1994; Ntwampe et al., 2010; Wang and Lan, 2018). However, there has been much speculation about the action mechanism(s) of Pluronics' interactions in cell cultures. It has been reported that Pluronic F68 physically interacts with insect cell cultures in a strong, direct and stable mode, since the shear protectant effects continue after its removal from the culture medium (Palomares et al., 2000). On the contrary, in cultures of the protozoa *Tetrahymena*, the multiple protectant effects conferred to Pluronic F68 disappeared after the surfactant was removed, indicating a low character of the surfactant-cell interactions (Hellung-Larsen et al., 2000). Therefore, the nature of the interactions between Pluronic F68 and cell surfaces seems to be dependent of the kind of cells assessed. In one of the very few studies reported on microalgae, Pluronic F68 showed to enhance the cell membrane integrity of *Chlamydomonas reinhardtii*. This surfactant is used in microalgal cultures as a shear protectant (Gallardo-Rodríguez et al., 2011; Ntwampe et al., 2010; Sobczuk et al 2006; Zhao et al., 2016), but the concentrations used are much lower than that employed in this study (lower than 2 CMC when used as cytoprotectant, while LF-PBR requires 12.5 CMC). Based on the existing knowledge, the precise mechanism of interaction between Pluronic F68 and algal cells covers seems to remain unknown, but it is beyond the scope of the present study.

Prior to cultivate microalgae in Pluronic F68-stabilized foam, the effect of this surfactant on liquid cultures of *Chlorella sorokiniana* was investigated in order to evaluate whether the interactions of the surfactant *per se* (not the surfactant-stabilized foam) result in altered cell biochemical composition. In this sense, Pluronic F68 was added to nutrient-replete and nitrogen-starved cultures in flasks (**Fig. 7.2**) at a concentration of 12.5 CMC, since this concentration was previously determined to be optimal for the liquid foam-bed photobioreactor operation (Chapter 5). The cultures were magnetically stirred and no additional CO_2 was supplied to avoid foam formation (see Materials and Methods, **Section 2.3.5**).

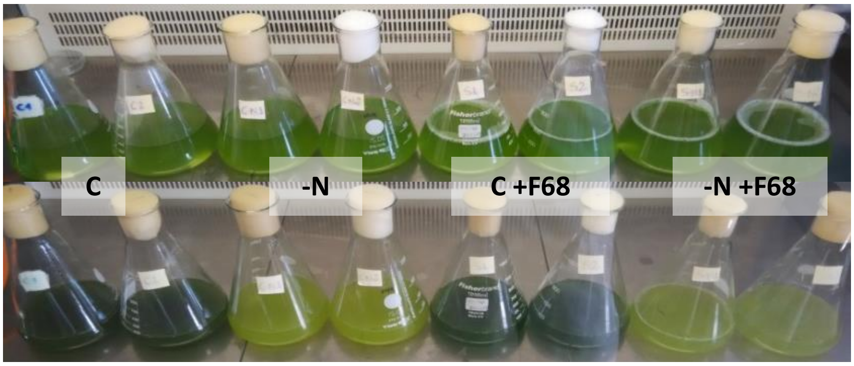


Figure 7.2. Liquid cultures of *Chlorella sorokiniana* at the beginning of the experiment (upper picture) and after 12 days of cultivation (lower picture). Culture conditions from left: control cultures in nutrient-replete culture medium without surfactant (C), nitrogen-starved cultures without surfactant (-N), cultures in nutrient-replete culture medium with the addition of Pluronic F68 (C +F68) and nitrogen-starved cultures with the addition of Pluronic F68 (-N +F68). The surfactant Pluronic F68 was added at 12.5 CMC (i.e. 4.175 g L^{-1}).

The growth of the cultures was measured in terms of optical density, dry weight and number of cells (**Fig. 7.3A-C**). As can be seen in the figures, the presence of Pluronic F68 did not affect *C. sorokiniana* growth in nutrient-replete or nitrogen-starved cultures. At the concentration used (i.e. 12.5 CMC), the lack of effects of this surfactant on *C. sorokiniana* growth in nutrient-replete conditions was known (Chapter 4). In addition to this, the results hereby presented proved the absence of effect on the growth of this alga also in nitrogen-starved liquid cultures. Pluronic F68 has shown growth-promoting effects in cultures of animal (Bentley et al., 1989) and plant cells (Kumar et al., 1992). However, reduced growth has also been observed in microalgal cultures (Camacho et al., 2007; Rodríguez et al., 2011) at much lower concentrations than that used in this study. Therefore, it seems that the effect of the surfactant on cell growth depends on the kind of culture and must be particularly assessed. As a result of the cytoprotectant effect of Pluronic F68, low concentrations of this surfactant have resulted in enhanced growth of mechanically stressed algal cultures (Sobczuk et al., 2006). However, no differences in the growth of nitrogen-starved cultures of *C. sorokiniana* were observed in our experiment.

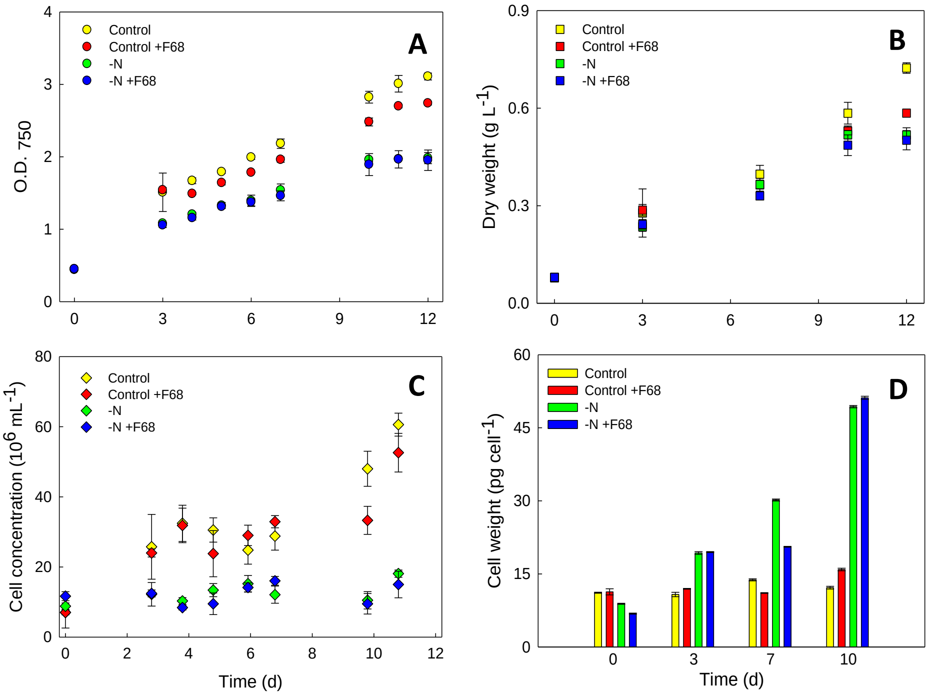


Figure 7.3. Time-course evolution of biomass concentration in terms of (A) optical density at 750 nm, (B) dry weight and (C) cell number; and (D) evolution of cell weight in liquid cultures of *Chlorella sorokiniana*. Culture conditions: nutrient-replete culture medium without surfactant (Control) and with the addition of Pluronic F68 (Control +F68), and nitrogen-free culture medium without surfactant (-N) and with the addition of Pluronic F68 (-N +F68). The surfactant Pluronic F68 was added at 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

On the other hand, all the nitrogen-starved cultures grew less than the control cultures, as expected. When nitrogen starvation was applied, different response of algal growth was observed in terms of optical density, cell number and dry weight. Optical density of nitrogen-starved cultures showed to increase during the first 10 days of cultivation, although it remained lower than that of nutrient-replete cultures (**Fig. 7.3A**). Similarly, dry weight of nitrogen-starved cultures increased during cultivation, however no differences among nutrient-replete and nitrogen-starved cultures were observed until day 10 (**Fig. 7.3B**). This is in accordance with the observations of Negi and co-workers (2015) on the continued growth of *C. sorokiniana* cultures up to 2 weeks after being subjected to nitrogen-deprivation. After 10 days of cultivation, small differences in the biomass concentration were observed, resulting in slightly lower maximal volumetric productivities in the nitrogen-starved cultures (**Table 7.2**).

Table 7.2. Growth and biochemical composition parameters of *Chlorella sorokiniana* cultivated in flasks. Culture conditions: nutrient-replete culture medium without surfactant (Control) and with the addition of Pluronic F68 (Control +F68), and nitrogen-free culture medium without surfactant (-N) and with the addition of Pluronic F68 (-N +F68). Maximal volumetric productivity (Max. Pv), maximal carbohydrates volumetric productivity (Max. Pv^{CH}), maximal carbohydrate to protein ratio (Max. CH/prot) and maximal carbohydrates to fatty acids ratio (Max. CH/FA). The surfactant Pluronic F68 was added at 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

	Assays in flasks			
	Control	**Control +F68**	**-N**	**-N +F68**
Max. Pv (g L^{-1} d^{-1})	0.07 ± 0.02	0.07 ± 0.01	0.05 ± 0.02	0.05 ± 0.02
Max. Pv^{CH} (g L^{-1} d^{-1})	0.01 ± 0.00	0.00 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
Max. CH/prot ratio	0.27 ± 0.20	0.53 ± 0.38	3.01 ± 0.14	3.01 ± 0.15
Max. CH/FA ratio	1.36 ± 0.17	1.90 ± 0.18	6.44 ± 0.16	5.50 ± 0.17

Conversely, the cell number of nitrogen-starved cultures remained nearly constant, in contrast to the nutrient-replete cultures which showed a cell number increase throughout the experimental time course (**Fig. 7.3C**). This is in accordance with many observations of slowed down cell division under nitrogen-starvation condition (Cakmak et al., 2012; Illman et al., 2000; Zhang et al., 2013). The biomass dry weight remained similar to those of the cultures under nutrients replenishment, but the cell number increase ceased, which indicates that the cell weight of nitrogen-starved cultures increased as can be observed in **Figure 7.3D**. An increase in the average cell size has been previously observed for *Chlorella* sp. and other microalgal strains as a result of nitrogen deprivation (Da Silva et al., 2009a; Yap et al., 2016).

Besides, the maximal photosynthetic efficiency of Photosystem II of the algal cells was measured along the experiment (**Fig. 7.4**). As expected from the previous results (Chapter 4), the presence of Pluronic F68 did not show any effect on the photosynthetic efficiency of non-stressed cultures, which remained constant along the experiment. On the other hand, the photosynthetic efficiency of all nitrogen-starved cultures dropped continuously during the experiment, as expected under nitrogen deprivation (Berges et al., 1996; Cheng et al., 2011). No differences in photosynthetic efficiency were found in the nitrogen-starved cultures due to the presence of the surfactant during the first week of cultivation; nonetheless, the presence of Pluronic F68 seemed to soften the photosynthetic efficiency decrease in the stressed cultures during the last days of experimentation. This may be related to the protective effects described for this surfactant. Pluronic F68 has shown to be an effective protectant against different mechanical and chemical stress, including nutrient starvation. Nevertheless, the particular protectant function in nutrient-deprived scenarios has been reported only for protozoa cells to date (Hellung-Larsen et al., 2000). In microalgal cultures, Pluronic F68 has shown to protect the cells against shear-associated damage (Camacho et al., 2007; Gallardo-Rodríguez et al., 2011; Sobczuk et al., 2006) and against toxic chemicals (Zhao et al., 2016), but there are no previous reports on its protective effect on nutrient-starved algal cultures.

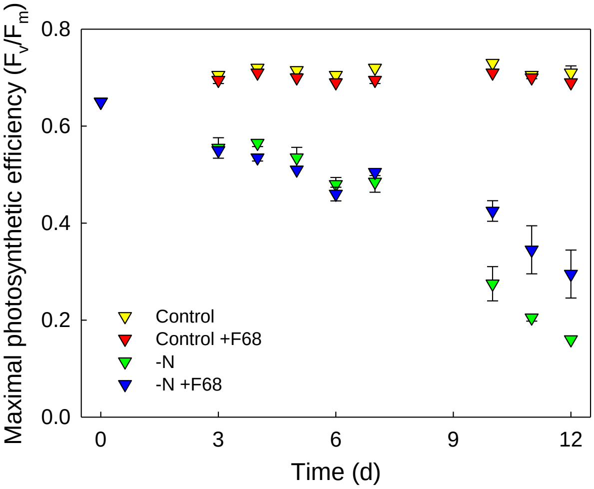


Figure 7.4. Maximal photosynthetic efficiency of Photosystem II (F_v/F_m) of *Chlorella sorokiniana* along cultivation in flasks. Culture conditions: nutrient-replete cultures without surfactant (Control) and with the addition of Pluronic F68 (Control +F68), and nitrogen-starved cultures without surfactant (-N) and with the addition of Pluronic F68 (-N +F68). The surfactant Pluronic F68 was added at 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

Next, whether the presence of the surfactant in nutrient-replete and nitrogen-starved liquid cultures may impact *Chlorella* biomass quality was investigated. For this, the major biochemical composition of the algal cells produced under the different conditions assayed was analyzed in terms of fatty acids, protein content, total carbohydrates and total pigments. Fatty acids were measured as fatty acid methyl esters (FAMEs) and the protein content was estimated from the nitrogen content measured by elemental composition analysis, using 4.5 as nitrogen-to-protein conversion factor (López et al., 2010) (see Materials and Methods, **Section 2.6.7**). The values of protein, carbohydrate and fatty acid content presented in this study correspond to the measurements done in the pellet after being washed with distilled water three times. The fatty acid content was also analyzed in the total broth in order to distinguish if there were fatty acids released into the supernatant of the cultures. The differences in the fatty acid content of pellet and total broth samples, this is the excretion of fatty acids during cultivation, are discussed in Chapter 9. Similarly, the presence of excreted carbohydrates was aim of study and carbohydrates were measured in both pellet and total broth samples. However, Pluronic F68 was shown to interfere with the carbohydrates measurement protocol by providing an additional signal. This was proved by measuring the carbohydrates in glucose solutions with Pluronic F68 at different concentrations (**Fig. 7.5**). As can be seen in the figure, increased Pluronic F68 concentrations resulted in increased carbohydrate signal, measured as absorbance at 483 nm. This effect was observed for solutions of glucose and Pluronic F68 at concentrations from 0 to 12 CMC and 0 to 0.1 g L^{-1}, respectively. Consequently, the possible excretion of carbohydrates could not be studied.

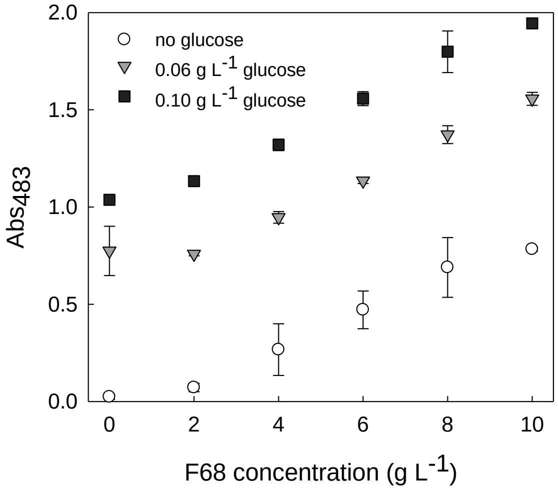


Figure 7.5. Carbohydrate signal expressed as absorbance at 483 nm of glucose solutions: no glucose, glucose at 0.06 g L^{-1} and glucose at 0.1 g L^{-1}; with the addition of Pluronic F68 at 0, 2, 4, 6, 8 and 10 g L^{-1}. The average values of samples analyzed at least in triplicate are shown, together with their corresponding standard deviations.

The major biochemical composition in terms of fatty acids, proteins, carbohydrates and pigments content of the cultures is shown in **Figure 7.6.** All the data were corrected considering the moisture and the ash content of the samples, which were in average 7.5% and 4.9% in the dry biomass, respectively. As can be seen in the figures, the algal cells did not experience changes in their biochemical composition along their cultivation in nutrient-replete medium, regardless the absence (**Fig. 7.6A**) or presence of the surfactant (**Fig. 7.6B**). The average major composition of *C. sorokiniana* in nutrient-replete medium was 12% of fatty acids, 47% of proteins, 13% of carbohydrates and 6% of pigments. On the other hand, nitrogen-starvation resulted in an increase of the carbohydrates content up to approximately 40%, while the protein fraction of the algal biomass decreased down to approximately 16%. It is well known that nitrogen starvation triggers the accumulation of fatty acids and carbohydrates in microalgae (Breuer et al., 2012; Ho et al., 2012). This can be explained by the transformation of microalgal proteins and peptides into storage compounds and also by *de novo* synthesis (Breuer et al., 2012; Ho et al., 2012). Besides, the pigment fraction also decreased in the nitrogen-deprived cultures to values lower than 1%, due to the lack of nitrogen to synthesize the chlorophyll molecules (Berden-Zrimec et al., 2008; Berges et al., 1996).

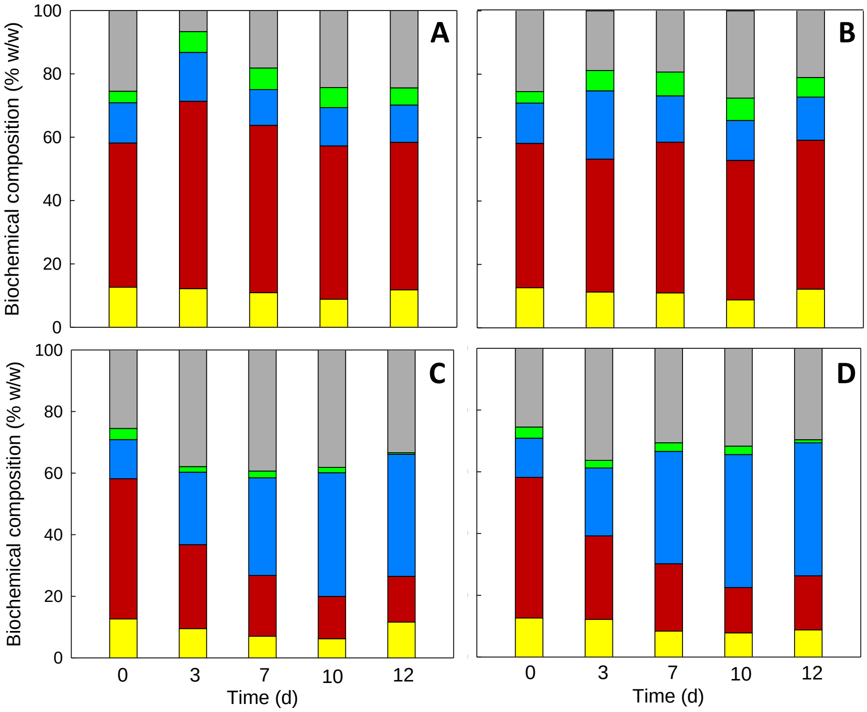


Figure 7.6. Evolution of the major biochemical composition of *Chlorella sorokiniana* liquid cultures in nutrient-replete culture medium (A) without surfactant and (B) with the addition of Pluronic F68, and in nitrogen-free culture medium (C) without surfactant and (D) with the addition of Pluronic F68. The surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). Major biochemical components: fatty acids (yellow), proteins (red), carbohydrates (blue), chlorophylls and carotenoids (green) and unknown compounds (grey). The average values of duplicate cultures are shown.

The increase in biomass concentration observed during nitrogen-starvation (**Fig. 7.3**) might be partly explained by the accumulation of carbohydrates as storage compounds. A high accumulation of carbohydrates (Cheng et al., 2017; John et al., 2011; Tanadul et al., 2014) or fatty acids (Breuer et al., 2012; Mujtaba et al., 2012) has been observed for different *Chlorella* strains. **Figure 7.6** illustrates that the lipid content of the nitrogen-starved *C. sorokiniana* cultures did not change. The changes observed in the biochemical composition resulted in approximately 7.5-fold and 4-fold higher carbohydrate to protein ratio and carbohydrate to fatty acid ratio under nitrogen deprivation, respectively (**Table 7.2**). Besides, and like in the non-stressed cultures, the major biochemical components in nitrogen-starved cultures evolved similarly regardless the presence of the surfactant. This indicates that the presence of Pluronic F68 did not have any effect on the overall composition of *C. sorokiniana* biomass, when cultivated in liquid cultures in either nutrient-replete or nitrogen-free medium.

Although nitrogen-starvation did not increase the fatty acid content of the biomass, it resulted in an increase of the fatty acid content per cell after 10 days (**Fig. 7.7A**), which correspond to the time when the maximal carbohydrate content is achieved (**Fig. 7.6C-D**). **Figure 7.7B** shows the time-course evolution of the fatty acid to carbohydrate ratio in the algal biomass. As can be seen, it decreases until day 10 in the nitrogen-starved cultures, after which it remained constant or even slightly increased. This would indicate that nitrogen-starved *Chlorella* cells accumulated both carbohydrates and fatty acids, but the production of carbohydrates occurred at higher rates than that of fatty acids during the first 10 days. After that, the cells might continue accumulating carbohydrates while probably converting part of those into fatty acids. Under nitrogen deprivation, an initial accumulation of carbohydrates and posterior conversion of those into fatty acids has been suggested for other microalgal strains such as *Chlamydomonas reinhardtii* (Cakman et al., 2012) and *Haematococcus pluvialis* (Recht et al., 2012).

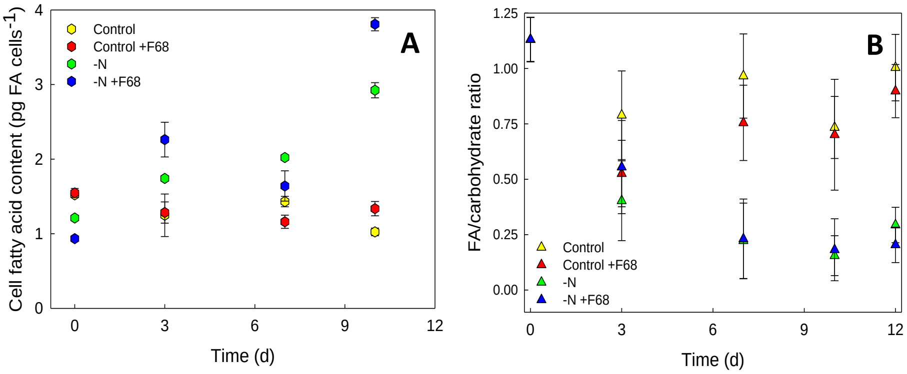


Figure 7.7. (A) Fatty acid content of *Chlorella sorokiniana* cells (pg fatty acids per cell) and (B) fatty acids to carbohydrate ratio (FA/carbohydrate ratio) of the biomass along cultivation in liquid cultures of *Chlorella sorokiniana*. Culture conditions: nutrient-replete culture medium without surfactant (Control) and with the addition of Pluronic F68 (Control +F68), and nitrogen-free culture medium without surfactant (-N) and with the addition of Pluronic F68 (-N +F68). The surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

Fatty acids are among the most valuable compounds produced by microalgae (Forján et al., 2015). In order to unravel the possible effect of the surfactant on the fatty acid composition of *C. sorokiniana* cells, the fatty acids profile was analyzed along the experiment. **Figure 7.8** shows a general fatty acid profile of *C.*

sorokiniana at the beginning of the experiment (t=0) and after 7 days of cultivation under the different conditions assessed. The percentage of saturated, monounsaturated (MUFA), polyunsaturated (PUFA) and unknown fatty acids from the total fatty acid content is shown. In general, a slight increase of saturated fatty acids was observed in the cultures under nitrogen starvation. In microalgal cultures, nitrogen starvation often results in a decrease in the average degree of unsaturation of the total fatty acid content (Breuer et al., 2012). A more detailed fatty acid composition of the cultures is shown in **Table 7.3**. It can be observed that the most abundant fatty acids are palmitic acid (C16:0), hexadecatrienoic acid (C16:3), linoleic acid (C18:2) and linolenic acid (C18:3). These are among the most abundant fatty acids previously found in *C. sorokiniana* (Chen and Johns, 1991; Negi et al., 2015; Ramanna et al., 2014). In particular, nitrogen deprivation showed to increase the relative abundance of palmitic and linoleic acids while decreasing that of linolenic acid (**Table 7.3**).

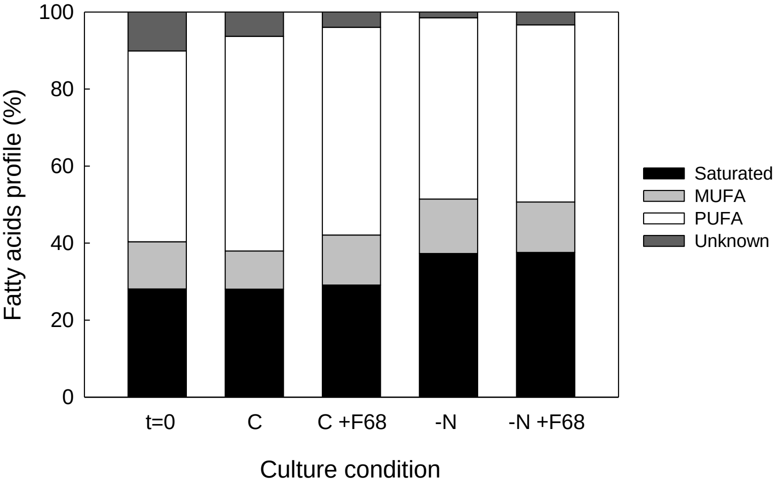


Figure 7.8. Fatty acid composition of *Chlorella sorokiniana* at day 0 (t=0) and after 7 days of cultivation in liquid cultures in flasks. Culture conditions: nutrient-replete culture medium without surfactant (C) and with the addition of Pluronic F68 (C +F68), and nitrogen-free culture medium without surfactant (-N) and with the addition of Pluronic F68 (-N +F68). The fatty acid composition is expressed as the percentage of saturated, monounsaturated (MUFA), polyunsaturated (PUFA) and unknown fatty acids. The surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown.

On the other hand, the presence of Pluronic F68 did not produce major alterations in the specific fatty acid composition of *C. sorokiniana* biomass in nutrient-replete or nitrogen-starved liquid cultures (**Table 7.3**).

Table 7.3. Fatty acid composition expressed as percentage of total fatty acids of *Chlorella sorokiniana* cultivated in batch mode in flasks. Culture conditions: nutrient-replete culture medium without surfactant (Control) and with the addition of Pluronic F68 (+Pluronic F68), and nitrogen-free culture medium without surfactant (N-starved) and with the addition of Pluronic F68 (N-starved +Pluronic F68). The average values of duplicate cultures are shown.

	Relative fatty acid abundance (%)											
	Flasks											
	Control			**+Pluronic F68**			**N-starved**			**N-starved +Pluronic F68**		
Time (d)	**0**	**3**	**7**	**0**	**3**	**7**	**0**	**3**	**7**	**0**	**3**	**7**
C13:0	0.21	0.00	0.00	0.21	0.00	0.00	0.21	0.00	0.00	0.21	0.00	0.00
C14:0	1.33	0.00	0.44	1.33	0.00	0.67	1.33	0.00	0.00	1.33	0.00	0.00
C14:1	0.90	0.36	0.00	0.90	0.00	0.46	0.90	0.00	0.00	0.90	0.00	0.00
C15:1	0.21	0.67	0.21	0.21	0.72	0.68	0.21	0.00	0.00	0.21	0.22	0.00
C16:0	20.65	23.04	23.73	20.65	25.05	23.96	20.65	27.29	30.41	20.65	29.10	30.87
C16:1	0.87	0.50	0.26	0.87	0.48	0.62	0.87	0.87	1.22	0.87	0.70	1.07
C16:2	0.92	3.44	3.10	0.92	3.08	3.37	0.92	2.04	1.33	0.92	1.68	1.82
C16:3	13.15	7.06	8.44	13.15	8.52	10.06	13.15	7.45	7.51	13.15	6.60	6.37
C17:0	0.29	0.55	0.00	0.29	0.17	0.00	0.29	0.33	0.00	0.29	0.69	0.00
C17:1	6.10	11.55	8.23	6.10	9.90	9.27	6.10	7.78	5.38	6.10	6.13	5.08
C18:0	5.39	5.75	3.25	5.39	7.12	4.49	5.39	8.42	6.55	5.39	15.53	6.72
C18:1n9c + C18:1n9t	4.17	2.30	0.93	4.17	2.08	1.94	4.17	5.30	7.54	4.17	2.41	6.94
C18:2n6c + C18:2n6t	24.53	18.13	21.63	24.53	19.18	22.57	24.53	22.15	28.46	24.53	18.77	27.23
C18:3n6	0.14	0.00	0.00	0.14	0.00	0.00	0.14	0.00	0.00	0.14	0.00	0.00
C18:3n3	10.82	20.59	17.10	10.82	17.81	17.95	10.82	13.56	9.79	10.82	11.31	10.55
C20:0	0.20	0.00	0.00	0.20	0.00	0.00	0.20	0.00	0.00	0.20	0.00	0.00
C20:2	0.00	0.00	1.74	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C20:5n3	0.00	0.00	1.82	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C22:0	0.00	0.00	0.61	0.00	0.00	0.00	0.00	0.00	0.34	0.00	0.00	0.00
C22:2	0.00	0.00	1.64	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C24:1n9 + C22:6n3	0.00	0.00	0.55	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Unknown	10.10	6.05	6.31	10.10	5.89	3.97	10.10	4.83	1.48	10.10	6.86	3.35
Saturated	28.08	29.34	28.03	28.08	32.34	29.12	28.08	36.03	37.31	28.08	45.32	37.59
MUFA	12.26	15.38	9.91	12.26	13.17	12.97	12.26	13.95	14.13	12.26	9.46	13.10
PUFA	49.55	49.23	55.75	49.55	48.60	53.94	49.55	45.19	47.08	49.55	38.36	45.96
Mufa/Pufa	24.73	31.24	17.77	24.73	27.11	24.05	24.73	30.86	30.01	24.73	24.66	28.49
n6/n3	227.98	88.07	112.73	227.98	107.68	125.76	227.98	163.35	290.74	227.98	165.89	258.15
n3	10.82	20.59	19.19	10.82	17.81	17.95	10.82	13.56	9.79	10.82	11.31	10.55
n6	24.66	18.13	21.63	24.66	19.18	22.57	24.66	22.15	28.46	24.66	18.77	27.23
n9	4.17	2.30	1.20	4.17	2.08	1.94	4.17	5.30	7.54	4.17	2.41	6.94

As above discussed, nitrogen deprivation resulted in a reduction of the total pigment content (**Fig. 7.6C-D**). This was due to the decrease of both chlorophyll and carotenoid content of the biomass (**Fig. 7.9B-D**). In general, the carotenoid content of the biomass increases under nitrogen deprivation (Minhas et al., 2016), but the present results show that nitrogen deprivation does not promote carotenoid accumulation in *C. sorokiniana.* Despite the biomass concentration increased under nitrogen starvation, a very little increase of chlorophyll concentration occurred (**Fig. 7.9A**), while the carotenoids concentration remained constant along cultivation and decreased after 7 days (**Fig. 7.9C**), leading to the total pigment content decrease observed in the biomass (**Fig. 7.6C-D**).

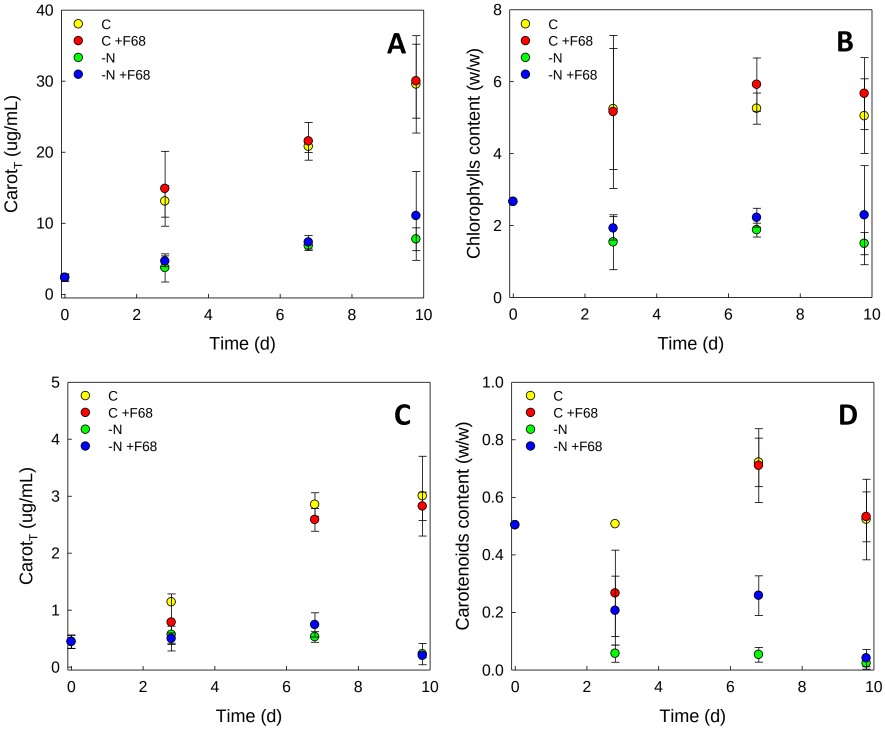


Figure 7.9. Evolution of (A) chlorophyll and (C) carotenoid concentration, expressed in µg mL^{-1}; and evolution of (B) intracellular chlorophyll and (D) carotenoid content during the cultivation of *Chlorella sorokiniana* in liquid cultures in flasks. Culture conditions: nutrient-replete culture medium without surfactant (C) and with the addition of Pluronic F68 (C +F68), and in nitrogen-free culture medium without surfactant (-N) and with the addition of Pluronic F68 (-N +F68). The surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

The methanolic extracts containing the photosynthetic pigments of *C. sorokiniana* were further analyzed by HPLC (High Performance Liquid Chromatography). Lutein is the major carotenoid in *C. sorokiniana* (Chen et al., 2015), and **Figure 7.10** shows the lutein content of the biomass at the beginning and after 10 days of cultivation. As can be observed in the figure, cultivation for 10 days under nitrogen deprivation resulted in a lutein content decrease down to half of the values obtained in nutrient–replete media. On the other hand, no differences in the content or the type of carotenoids were found due to the presence of the surfactant in either nutrient-replete or nitrogen-starved cultures.

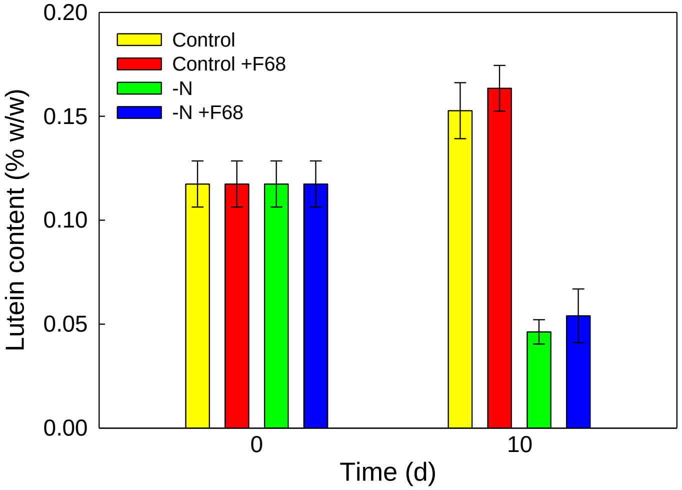


Figure 7.10. Lutein content of *Chlorella sorokiniana* (% w/w) at day 0 and after 10 days of cultivation in liquid cultures in flasks. Conditions: nutrient-replete culture medium without surfactant (Control) and with the addition of Pluronic F68 (Control +F68), and nitrogen-free culture medium without surfactant (-N) and with the addition of Pluronic F68 (-N +F68). The surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

In general, the results presented above indicate the lack of effects of Pluronic F68 on the growth and the biochemical composition of *C. sorokiniana* in both nutrient-replete and nitrogen-starved liquid cultures.

7.3.3 Pluronic F68 effects on foam-based cultures

Once the effects of Pluronic F68 on the biochemical composition of *C. sorokiniana* were determined in liquid cultures in flasks, they were also analyzed in cultures performed in the liquid foam-bed photobioreactor (LF-PBR). The data obtained from foam-based cultures were compared with reference data from a base case: *C.*

sorokiniana grown as liquid culture in the LF-PBR. The liquid base case and the foam-based control cultures performed in the LF-PBR were operated under the conditions shown in **Table 7.4**. Each culture was performed in duplicate, using two LF-PBR units that were operated in batch mode for 7 days.

Table 7.4. Operational parameters of the liquid base case cultures and the foam-based control cultures performed in the liquid foam-bed photobioreactor.

Parameter	Liquid, base case	Foam, control
Culture medium	M-8	M-8
Surfactant and concentration	-	Pluronic F68, 12.5 CMC
Gas flow rate	3000 mL min^{-1}	3000 mL min^{-1}
Superficial gas flow rate	2.5 10^{3} m s^{-1}	2.5 10^{3} m s^{-1}
CO_2 enrichment	2.71% (v/v)	2.71% (v/v)
Illumination system	Fluorescent lamps panel	Fluorescent lamps panel

Figure 7.11 shows the microalgal growth in terms of optical density (**Fig. 7.11A**) and dry weight (**Fig. 7.11B**). As can be observed, much larger growth was found in foam-based cultures than in the corresponding liquid cultures in the LF-PBR. Biomass productivity values of 2.2 g L^{-1} d^{-1} were obtained in M-8 culture medium in the foam-based cultures, which is approximately 7-fold higher than those obtained in liquid cultures in the LF-PBR (0.32 g L^{-1} d^{-1}) (**Table 7.2**).

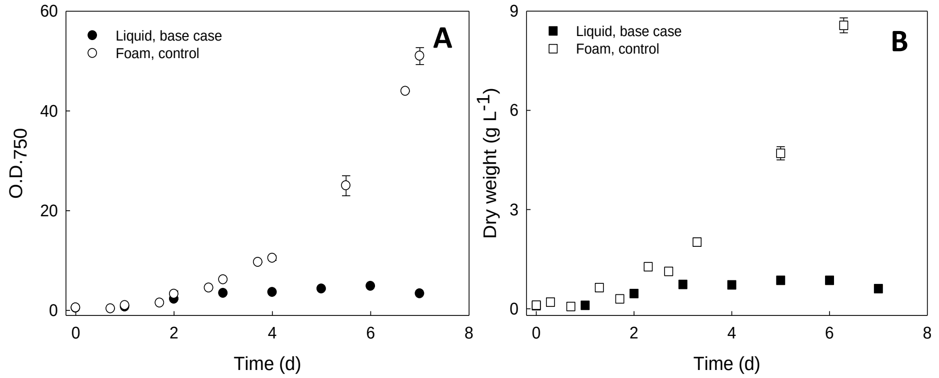


Figure 7.11. Time-course evolution of the biomass concentration in liquid (Liquid, base case) and foam-based (Foam, control) cultures of *Chlorella sorokiniana* in standard M-8 culture medium in the liquid foam-bed photobioreactor, in terms of (A) optical density at 750 nm and (B) dry weight. The average values of duplicate cultures are shown, together with their corresponding standard deviations.

Table 7.5. Growth and biochemical composition parameters of *Chlorella sorokiniana* cultivated in the liquid foam-bed photobioreactor. Culture conditions: liquid cultures (Liquid, base case), foam-based cultures in standard M-8 culture medium (Foam, control), foam-based cultures in 3-fold concentrated M-8 culture medium (Foam, 3-fold medium), foam-based culture in nitrogen-free M-8 culture medium (Foam, N-starved) and foam-based culture in 3-fold concentrated M-8 culture medium under saturating light (Foam, saturating light). Maximal volumetric productivity (Max. Pv), maximal carbohydrates volumetric productivity (Max. Pv^{CH}), maximal carbohydrate to protein ratio (Max. CH/prot) and maximal carbohydrate to fatty acids ratio (Max. CH/FA ratio). In all foam-based cultures, the surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}).

	Assays in the liquid foam-bed reactor				
	Liquid, base case	**Foam, control**	**Foam, 3-fold medium**	**Foam, N-starved**	**Foam, saturating light**
Max. Pv ($g\ L^{-1}\ d^{-1}$)	0.32 ± 0.05	2.19 ± 0.04	4.00 ± 1.49	0.05 ± 0.01	5.95 ± 0.75
Max. Pv^{CH} ($g\ L^{-1}\ d^{-1}$)	0.01 ± 0.01	0.68 ± 0.16	2.23 ± 0.22	0.00 ± 0.00	0.54 ± 0.05
Max. CH/prot ratio	0.30 ± 0.09	1.38 ± 0.04	1.14 ± 0.07	2.75 ± 0.14	0.54 ± 0.03
Max. CH/FA ratio	1.56 ± 0.07	5.21 ± 0.09	5.93 ± 0.08	7.46 ± 0.30	2.85 ± 0.03

The evolution of the surfactant concentration was measured in the foam-based cultures and compared to the data obtained during the operation of the LF-PBR with only Pluronic F68-added culture medium (Chapter 6). It was previously observed that in the LF-PBR operated with the culture medium containing the surfactant the concentration of Pluronic F68 decreases probably due to the attachment of the surfactant to the system (Chapter 6). Although no strong attachment of the Pluronic molecules to *Chlorella* cells was observed in liquid cultures (**Fig. 7.1**), the results presented in **Figure 7.12** illustrate that the concentration of Pluronic F68 in the LF-PBR decreased slightly faster in the presence of biomass. This might be due to physical interactions between the surfactant molecules and the algal cells, or to an enhanced attachment of the surfactant to the system, since part of the biomass also stuck to the system during its cultivation in the LF-PBR.

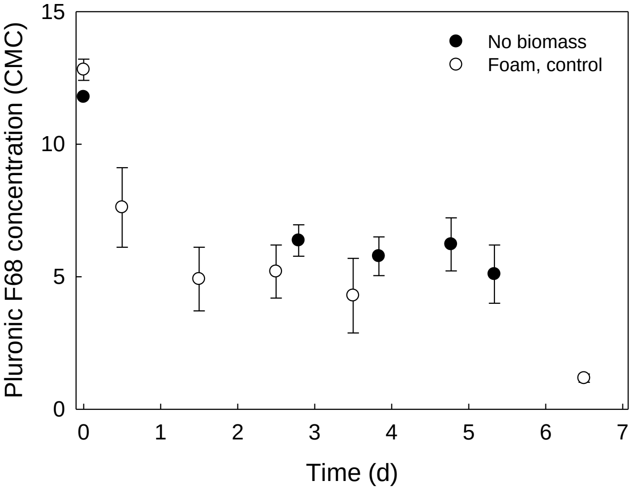


Figure 7.12. Pluronic F68 concentration (CMC) in the total liquid operated with Pluronic F68 solution (No biomass) and during the foam-bed cultivation of *Chlorella sorokiniana* in standard M-8 culture medium supplemented with Pluronic F68 (Foam, control). The surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of experimental duplicates are shown, together with their corresponding standard deviations.

In order to determine the effects of cultivation in the LF-PBR on *Chlorella* biomass quality, the major biochemical composition of the cultures was analyzed and it is shown in **Figure 7.13**. The biochemical composition in biomass samples taken from both liquid and foam-based cultures was similar until day 3, but significantly different at the end of cultivation. Whereas the composition of biomass samples from standard liquid cultures remained unchanged all along the cultivation time, the samples from foam-based cultures experienced a large increase in the carbohydrates fraction (from 6% to 42%) and also a significant decrease in the protein fraction (from 56.5% to 30.5%) after 7 days of cultivation. In contrast, the lipid fraction (namely fatty acids and pigment contents) showed similar evolution trends in time (slight decrease) in both foam and liquid cultures.

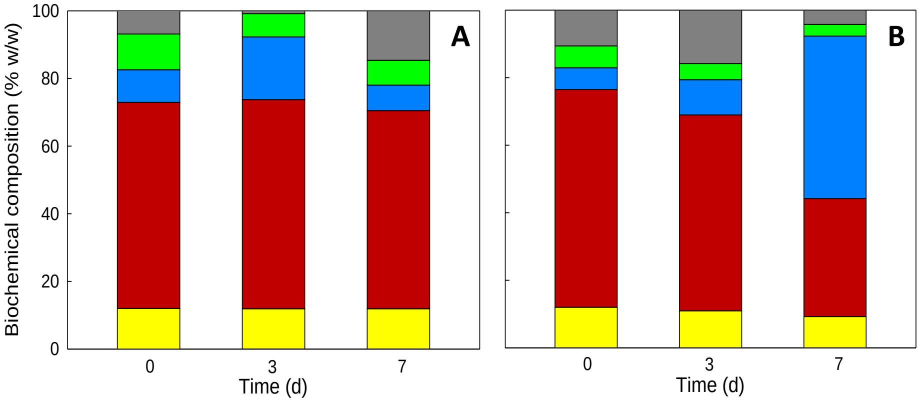


Figure 7.13. Major biochemical composition of (A) liquid base case, and (B) foam-based control cultures of *Chlorella sorokiniana* cultivated in standard M-8 culture medium in the liquid foam-bed photobioreactor operated in batch mode. The surfactant Pluronic F68 was added to the foam-based control cultures at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). Major biochemical components: fatty acids (yellow), proteins (red), carbohydrates (blue), chlorophylls and carotenoids (green) and unknown compounds (grey). The average values of duplicate cultures are shown.

The noticeable increase in the carbohydrate fraction of algal biomass produced in stabilized foam is consistent with imbalanced nitrogen assimilation with respect to carbon assimilation, as previously observed in nitrogen-starved liquid cultures (**Fig. 7.6C-D**). This may obviously reflect limitation (or absence) of inorganic nitrogen as a primary consequence of fast algal growth in the foam-based cultures. Under nitrogen limitation or starvation, the carbon metabolic fluxes of the algal cells are mostly addressed to the biosynthesis of carbon and energy resources. As previously discussed, it is well known that in nitrogen-starved cultures of microalgae, cell division ceases and carbon partitioning is first redirected towards the biosynthesis of carbohydrates including polysaccharides (namely starch, Zachleder and Brányiková 2014). Among the many examples found in literature, cultures of *Scenedesmus obliquus* and *Chlorella zofingiensis* grown under cultivation conditions –light intensity and CO_2 (% v/v)- similar to those values in our experimental set-up, yielded carbohydrate productivities of about 0.4

g L^{-1} d^{-1} and abundances of 56% in average when nitrogen was starved (Cheng et al., 2017; Ho et al., 2012; Zhu et al., 2014). The results shown in **Table 7.5** evidence a high carbohydrate productivity of 0.68 g L^{-1} d^{-1} in foam-based control cultures (using standard M-8 culture medium) by the end of cultivation (day 7), compared to the almost null net carbohydrate production in the liquid base case cultures in the photobioreactor. Consistently with these results, the carbohydrate to protein ratio in biomass of foam-based cultures on day 7 is about 4.5-fold higher than that of liquid cultures. Thus, the obtained results for *C. sorokiniana* grown in foam suggest a N-starvation typical scenario at the end of the cultivation period, also consistent with the accumulation of carbohydrates prior to that of lipids, which is evidenced by the increased carbohydrate to fatty acids ratio in the foam-based cultures by day 7 (**Table 7.5**).

Regarding the lipid fraction, despite the fatty acid content of the biomass cultivated in the LF-PBR did not increase, differences in the fatty acid profile were observed with respect to the biomass cultivated in liquid and in the presence of Pluronic F68. (**Fig. 7.8** and **7.14**; **Table 7.3** and **7.6**). *C. sorokiniana* biomass cultivated in the LF-PBR showed, in general, an increased relative abundance of total unsaturated fatty acids as well as a higher monounsaturated/polyunsaturated fatty acids ratio (MUFA/PUFA).

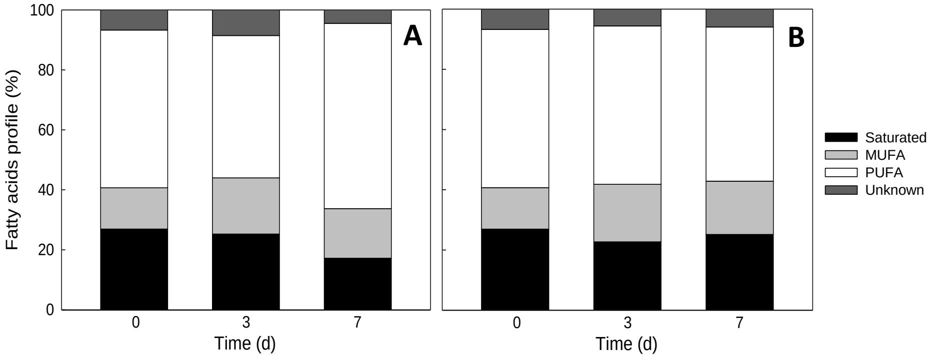


Figure 7.14. Fatty acid composition of *Chlorella sorokiniana* in (A) liquid base case, and (B) foam-based control cultures, in standard M-8 culture medium in the liquid foam-bed photobioreactor (LF-PBR). The fatty acid composition is expressed as the percentage of saturated, monounsaturated (MUFA), polyunsaturated (PUFA) and unknown fatty acids. The surfactant Pluronic F68 was added to the foam-based cultures at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown.

Table 7.6. Fatty acid composition expressed as percentage of total fatty acids of *Chlorella sorokiniana* cultivated in batch mode in the liquid foam-bed photobioreactor. Culture conditions: liquid cultures (Liquid, base case), foam-based cultures in standard M-8 culture medium (Foam, control), foam-based cultures in 3-fold concentrated M-8 culture medium (Foam, 3-fold medium), foam-based culture in nitrogen-free M-8 culture medium (Foam, N-starved) and foam-based culture in 3-fold concentrated M-8 culture medium under saturating light (Foam, saturating light). The average values of duplicate cultures are shown.

	Relative fatty acid abundance (%)																		
	Liquid foam-bed photobioreactor																		
	Liquid, base case			Foam, control			Foam, 3-fold medium					Foam, N-starved			Foam, saturating light				
Time (d)	**0**	**3**	**7**	**0**	**3**	**7**	**0**	**3**	**7**	**10**	**13**	**0**	**3**	**7**	**0**	**3**	**7**	**10**	**13**
C13:0	0.26	0.80	0.27	0.00	0.00	0.00	1.16	1.69	1.24	1.03	0.51	0.35	0.00	0.00	1.30	0.75	0.00	0.78	0.58
C14:0	1.54	1.35	0.79	0.58	0.98	0.71	0.45	0.65	0.51	0.39	0.19	1.12	0.68	0.55	0.72	0.42	0.86	1.23	0.33
C14:1	0.97	0.89	0.44	0.78	1.26	0.90	0.69	0.90	0.80	0.64	0.34	0.66	0.47	0.26	0.58	0.70	0.76	0.77	0.55
C15:1	0.27	0.83	0.36	0.34	0.85	0.55	1.31	1.67	1.29	1.11	0.56	9.71	0.34	0.44	0.68	0.88	0.63	0.96	0.63
C16:0	16.77	18.19	15.35	23.34	18.10	22.44	16.91	14.75	20.92	20.34	22.14	10.13	26.41	29.06	18.12	17.41	19.35	19.46	17.53
C16:1	0.69	0.61	0.45	0.31	0.00	0.43	0.63	0.41	0.75	0.58	0.51	0.80	0.61	0.61	0.53	0.72	0.92	0.86	0.76
C16:2	1.09	1.24	1.11	0.32	2.97	1.22	1.39	2.33	4.92	1.00	0.58	6.04	1.31	0.88	0.82	2.00	1.68	1.49	1.25
C16:3	12.63	6.67	13.88	13.39	7.87	10.16	9.82	8.20	4.59	9.35	6.64	5.78	5.78	5.33	5.54	8.83	10.16	11.70	10.73
C17:0	0.22	0.37	0.00	0.25	0.00	0.00	0.00	0.00	0.37	0.00	0.23	6.00	0.46	0.51	0.35	0.22	0.00	0.00	0.00
C17:1	11.31	13.67	12.67	9.07	13.29	8.48	13.98	13.14	9.96	8.66	7.64	8.96	9.73	7.01	9.07	12.70	10.79	11.35	10.04
C18:0	2.36	4.58	0.81	8.57	3.62	2.01	3.78	3.80	9.46	13.64	19.77	4.35	5.55	12.94	11.47	5.85	5.03	6.59	7.64
C18:1n9c + C18:1n9t	2.41	2.68	2.56	1.32	3.71	7.33	0.00	0.00	0.00	0.00	0.00	11.02	7.47	7.49	0.00	0.00	2.71	0.00	0.00
C18:2n6c + C18:2n6t	22.11	16.34	27.17	23.34	18.28	26.02	19.42	18.99	18.71	20.13	18.79	9.81	19.94	17.87	13.51	19.94	20.42	21.67	21.82
C18:3n6	0.00	0.00	0.10	0.28	0.00	0.00	0.00	0.00	0.33	0.00	0.34	9.24	0.00	0.00	0.00	0.00	0.21	0.00	0.00
C18:3n3	17.67	23.19	19.56	14.34	23.45	13.77	22.63	23.33	17.17	14.55	12.74	9.46	15.89	11.35	14.44	22.03	20.20	20.22	17.98
C20:0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.27	0.43	0.00	0.00	0.00	0.00
C20:2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.23	0.00	0.53	0.00	0.61
C20:5n3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.06	0.00	0.50	0.00	0.45
C22:0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.44	0.82	0.00	0.00	0.00
C22:2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.76	0.00	0.49	0.00	0.47
C24:1n9 + C22:6n3	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.30	0.00	0.37	0.00	0.31
Unknown	9.69	8.57	4.48	3.78	5.61	5.98	7.85	10.15	8.99	8.58	9.01	6.56	5.37	5.42	14.66	6.74	4.38	2.93	8.32
Saturated	21.16	25.29	17.21	26.94	22.71	25.17	22.29	20.88	32.50	35.40	42.84	21.95	33.10	43.34	32.82	25.47	25.24	28.06	26.08
MUFA	15.66	18.70	16.49	11.82	19.11	17.69	16.60	16.13	12.80	10.99	9.06	31.15	18.61	15.81	11.50	15.00	16.00	13.93	12.13
PUFA	53.50	47.45	61.82	51.67	52.58	51.16	53.26	52.84	45.71	45.03	39.10	40.33	42.92	35.44	41.02	52.79	54.38	55.07	53.47
Mufa/Pufa	29.26	39.40	26.68	22.87	36.33	34.57	31.17	30.52	28.00	24.40	23.17	77.24	43.37	44.60	28.04	28.41	29.42	25.30	22.69
n6/n3	125.10	70.46	139.43	164.71	77.96	188.89	85.83	81.43	110.87	138.35	150.18	201.38	125.51	157.47	78.78	90.50	98.77	107.17	117.44
n3	17.67	23.19	19.56	14.34	23.45	13.77	22.63	23.33	17.17	14.55	12.74	9.46	15.89	11.35	17.15	22.03	20.89	20.22	18.58
n6	22.11	16.34	27.27	23.61	18.28	26.02	19.42	18.99	19.03	20.13	19.14	19.05	19.94	17.87	13.51	19.94	20.63	21.67	21.82
n9	2.41	2.68	2.56	1.32	3.71	7.33	0.00	0.00	0.00	0.00	0.00	11.02	7.47	7.49	0.65	0.00	2.89	0.00	0.15

In order to determine whether nitrogen (added as urea) was rapidly depleted thus leading to the growth cease of *C. sorokiniana* in foam, two LF-PBR were run in batch mode with 3-fold concentrated culture medium (two LF-PBR units operating in parallel as culture duplicates). This means that the initial urea concentration was 3-fold higher than the one used in the previous experiments. The batches were prolonged for 13 days by punctually adding surfactant in order to maintain the foaming (**Fig. 7.15**). As described in the previous Chapter, Pluronic F68 sticks to the LF-PBR, thus leading to a decrease in the solution foamability. Hence, the surfactant concentration was measured daily after correcting the evaporation, and when a decrease in the foamability was observed, the corresponding amount of surfactant needed to keep a concentration of 12.5 CMC was added. In this experiment, 2.15 and 3 grams of extra surfactant was added per LF-PBR unit (of a total of two units) after 3 and 6 days of cultivation, respectively. Besides, liquid evaporation was measured and corrected with distilled water daily. Liquid evaporation rate was in average 121±20 mL d^{-1} per LF-PBR unit.

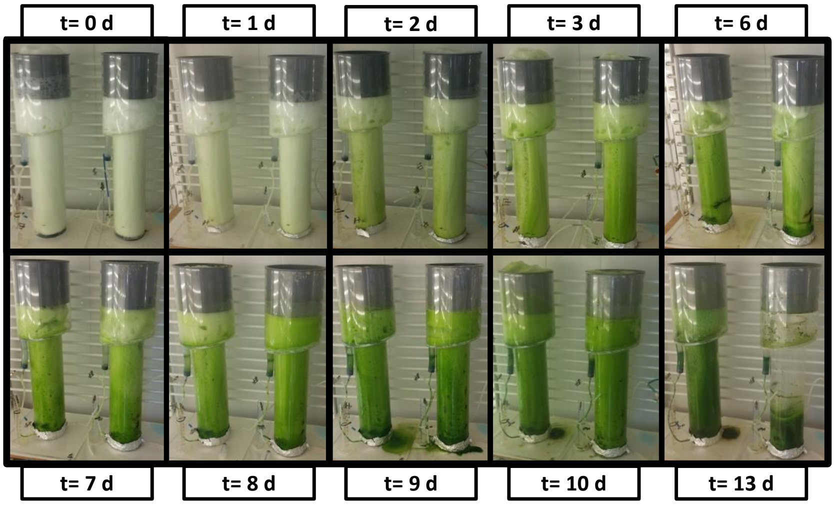


Figure 7.15. *Chlorella sorokiniana* along the foam-based cultivation in batch mode in the foam-bed photobioreactors in 3-fold concentrated culture medium.

The growth of *C. sorokiniana* in foam-based cultures in 3-fold concentrated culture medium is shown in **Figure 7.16**. During the first week, the microalgal growth was similar to that of the foam-based cultures in non-concentrated culture medium (**Fig. 7.11B**). However, the increase in nutrients concentration and the subsequent prolonged cultivation period of *Chlorella* might be among the reasons to explain the higher biomass concentrations (23 g L^{-1}) as well as the higher (almost twice) maximal volumetric biomass productivity achieved compared to the results obtained in standard culture medium (**Table 7.5**).

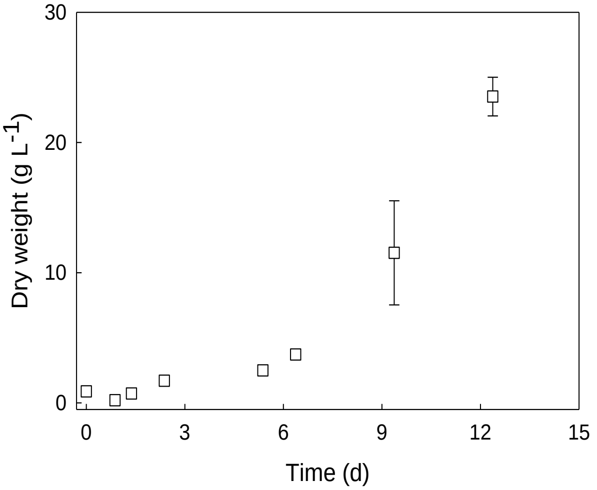


Figure 7.16. Time-course evolution of the biomass concentration in foam-based cultures of *Chlorella sorokiniana* in 3-fold concentrated M-8 culture medium in the liquid foam-bed photobioreactor expressed in terms of dry weight (g L^{-1}). The surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

Volumetric biomass productivities up to 12 g L^{-1} d^{-1} have been reported for *C. sorokiniana* using optimized cultivation parameters including illumination, dilution factor, temperature and heterotrophic cultivation (Cuaresma et al., 2009; Li et al., 2013). Nevertheless, the biomass productivities achieved by *C. sorokiniana* cultures in foam-based cultures (2.2 and 4 g L^{-1} d^{-1} with standard and 3-fold culture media, respectively) were high compared to that obtained by this strain in other cultivations systems such as tubular reactors (Ugwu et al., 2005). Moreover, considering that the cultivation conditions in the algal room were far from optimal values, our results in foam suggest this novel cultivation concept might be a promising tool for algal biomass production.

The question why the algal growth in foam might perform at even higher rates than in liquid cultures and thus achieving increased biomass concentrations should be answered from different approaches: (i) increased gas transfer in the foam (Janoska et al., 2018a), particularly CO_2 transfer from the air bubbles inside the foam to the algal cells contained in the thin liquid layers that surround them; and (ii) possible increased cell membrane permeability to nutrients that results in higher nutrients consumption rates (Taoka et al., 2011).

The algal growth in foam might be enhanced by the surfactant-mediated increased cell membrane permeability. Non-ionic surfactants (Pluronic F68 included) are, in general, less toxic to growing microalgae than the cationic or anionic ones (Chapter 4) and thus might be employed to modulate effects on the algal cells response. For instance, in one of the very few examples found in literature, the non-ionic surfactant polyoxyethylene sorbitanmonooleate (Tween 80) was shown to improve nutrient availability to microorganism cells by improving cell membrane permeability. Taoka et al. (2011) found that addition of 1% (v/v) Tween 80 to the culture medium of *Thraustochytrium aureum* ATCC 34304 (marine protist) resulted in 2-fold increase in biomass and 1.15-fold increase in lipid content. The possibility exists that (i) the surfactant induces changes in the algal physiology based on its interaction with molecules of the cell cover, and/or (ii) the physicochemical conditions of the microenvironment around the algal cells (improved mass transfer -particularly for carbon dioxide-, increased light availability) in the thin liquid layers between foam bubbles alter the algal physiology and growth. Whether the positive effect on the algal growth relies on the improved permeability of the cell membrane by the surfactant or on the increased mass transfer and light availability remains to be investigated.

Regarding the biochemical composition of the biomass, the carbohydrate content was again found to increase, mostly at the expense of proteins (**Fig.7.17A**). In this case, the biochemical changes occurred after about 2 weeks of cultivation. It happened in coincidence with nitrogen concentration decreasing to limiting levels, as can be seen in **Figure 7.17B**. The concentration of urea, the only nitrogen source in these cultures, was measured along cultivation by using an enzymatic kit (see Materials and Methods, **Section 2.6.9**). Urea was completely depleted by day 13. The rapid nitrogen depletion from the culture medium and the very high biomass concentrations achieved in the LF-PBR operated in batch mode are the result of the fast algal growth in foam-based cultures when compared to that in the liquid cultures of *C. sorokiniana* under the same cultivation conditions (**Fig.7.11**, **Table 7.5**).

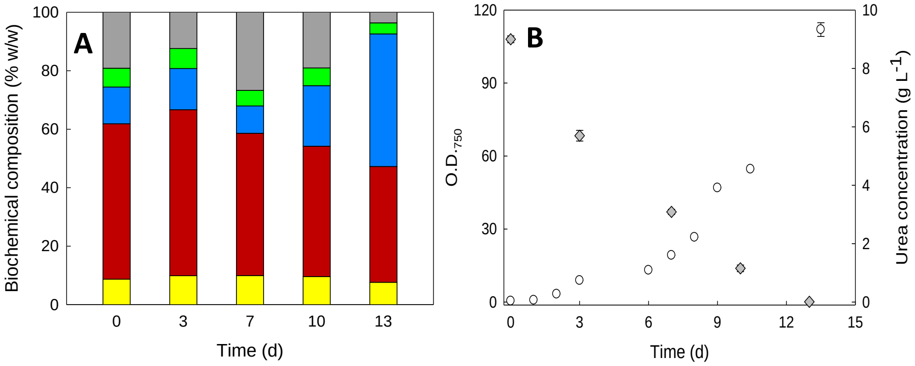


Figure 7.17. Foam-based cultures of *Chlorella sorokiniana* batch-cultivated in the liquid foam-bed photobioreactor in 3-fold concentrated M-8 culture medium. (A) Evolution of the major biochemical composition: fatty acids (yellow), proteins (red), carbohydrates (blue), chlorophylls and carotenoids (green) and unknown compounds (grey). (B) Time-course evolution of the biomass concentration in terms of optical density at 750 nm (white dots), and urea concentration (grey rhombus). The surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

The maximal photosynthetic efficiency also seemed to decrease at the time urea was depleted and the cells experienced a nitrogen-starvation scenario (**Fig. 7.18**). The lack of nitrogen results in a rapid decrease of the photosynthetic efficiency of the photosystem II (PSII) in microalgae (Ruíz-Domínguez et al., 2015), due to the loss of chlorophyll a and the decrease in PSII proteins synthesis (Berden-Zrimec et al., 2008). The time-course evolution of the maximal photosynthetic efficiency of PSII of *C. sorokiniana* cultivated in the LF-PBR in standard culture medium in liquid and foam-based cultures, and in 3-fold concentrated culture medium in foam-based cultures is shown in **Figure 7.18**. As can be seen, the photosynthetic efficiency of *Chlorella* cells remained unchanged in liquid cultures. Little growth was found in these liquid cultures (**Fig. 7.11**) and no changes in the biochemical composition of the biomass were found (**Fig. 7.13A)**, indicating that nutrients were still available at the end of the experimentation. Foam-based cultures in standard culture medium showed larger growth (**Fig. 7.11**) and a decrease of the photosynthetic efficiency after 6 days of cultivation (**Fig. 7.18**). At this time of the experiment, most of the urea had probably been consumed, thus the cultures entered already in a nitrogen starvation scenario, probably resulting in the biochemical composition changes previously described (**Fig. 7.13B**). However, by increasing nutrients in the culture medium of foam-based cultures, the maximal photosynthetic efficiency remained constant until day 10, after which urea was consumed (**Fig. 7.17B**) and the photosynthetic efficiency started to decrease (**Fig.**

7.18). The consumption of urea also coincided with the changes in the major biochemical composition previously described (carbohydrates increase, N-derived compounds decrease) (**Fig. 7.17A**).

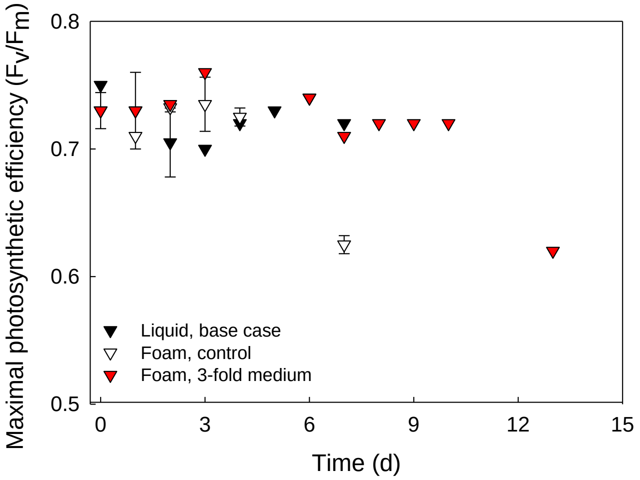


Figure 7.18. Maximal photosynthetic efficiency of Photosystem II (F_v/F_m) of *Chlorella sorokiniana* along cultivation in the foam-bed photobioreactor. Cultivation conditions: liquid cultures in standard M-8 culture medium (Liquid, base case), foam-based cultures in standard M-8 culture medium (Foam, control) and foam-based cultures in 3-fold concentrated M-8 culture medium (Foam 3-fold medium). The surfactant Pluronic F68 was added to the foam-based control cultures at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

Although the lipid fraction did not change during cultivation (**Fig. 7.17A**), the fatty acid profile showed a continuous increase of the relative abundance of saturated fatty acids, which increased up to 40% (**Fig. 7.19**). The most drastic changes were observed for the C18 fatty acids; stearic acid (C18:0) relative abundance increased from approximately 4% up to 20% in detriment of the relative abundance of linolenic acid (omega-3 fatty acid C18:3n3), which decreased from approximately 23% to 13% (**Table 7.6**).

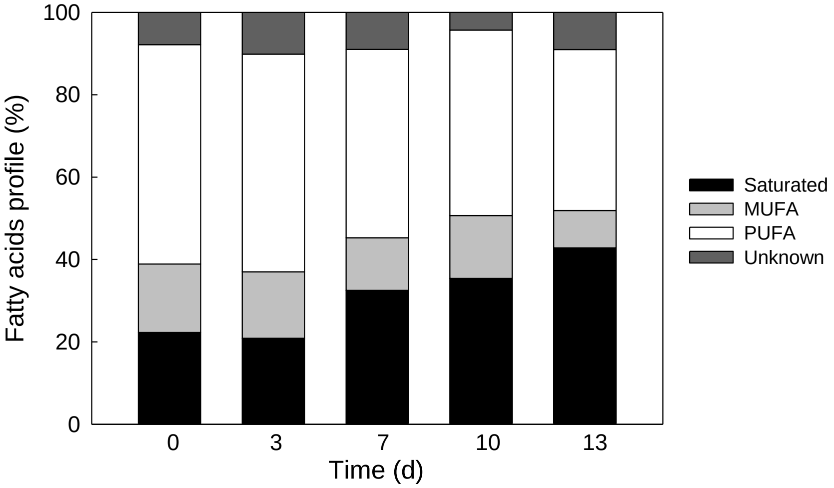


Figure 7.19. Fatty acid composition of *Chlorella sorokiniana* in foam-based cultures in 3-fold concentrated M-8 culture medium in the liquid foam-bed photobioreactor. The fatty acid composition is expressed as the percentage of saturated, monounsaturated (MUFA), polyunsaturated (PUFA) and unknown fatty acids. The surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown.

Furthermore, nitrogen-starved foam-based cultures of *C. sorokiniana* were performed in foam in the LF-PBR in order to analyze whether the microalgae produced in foam and subjected to stress conditions showed similar responses to those of microalgae produced in liquid cultures. This might have implications to design stress strategies for target compounds accumulation in microalgae produced in surfactant-stabilized foam. The evolution of biomass concentration and maximal photosynthetic efficiency of *C. sorokiniana* in nitrogen-starved foam-based cultures, compared to that in nitrogen-starved liquid cultures with the presence of the surfactant, is shown in **Figure 7.20**.

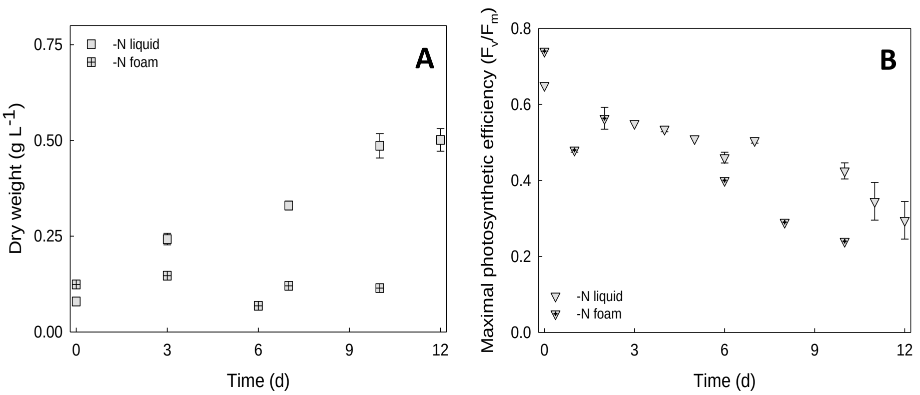


Figure 7.20. *Chlorella sorokiniana* batch-cultivated in flasks (-N liquid) and in the liquid foam-bed photobioreactor (-N foam) in nitrogen-free M-8 culture medium with the addition of Pluronic F68. (A) Time-course evolution of the biomass concentration in terms dry weight (g L^{-1}). (B) Maximal photosynthetic efficiency of Photosystem II (F_v/F_m) of *Chlorella sorokiniana* along cultivation. The surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

It can be observed in **Figure 7.20A** that foam-based cultures ceased growing immediately when subjected to nitrogen starvation, while liquid cultures continued increasing in dry weight (due to the increase of cellular weight, **Fig 7.3D**). Accordingly, the photosynthesis inhibition of algal cells growing in foam occurred at a faster rate than that in nitrogen-starved liquid cultures (**Fig. 7.20B**).

Besides, the changes observed in the biochemical composition of the biomass (**Fig. 7.21**) followed a similar pattern than that in liquid cultures under nitrogen starvation (**Fig. 7.6D**). The carbohydrate fraction increased (from approximately 20% up to 50% from day 3 to 10) in detriment of the protein fraction of the algal biomass, which decreased from 45% to 15%, approximately. The pigment content also decreased drastically, since the lack of nitrogen limits the synthesis of chlorophylls. The lipid content in algal biomass of foam-based cultures remained unchanged until the end of the experiment in the batch systems (about 10 days), probably due to the sequential accumulation of carbohydrates and lipids –this order- in nitrogen-starved *C. sorokiniana* cultures. The increase in the carbohydrate fraction of algal biomass in N-starved foam-based cultures (**Fig. 7.21**) was slightly faster and significantly greater (in 10 days) than that found in nitrogen-starved liquid cultures (**Fig. 7.6D**).

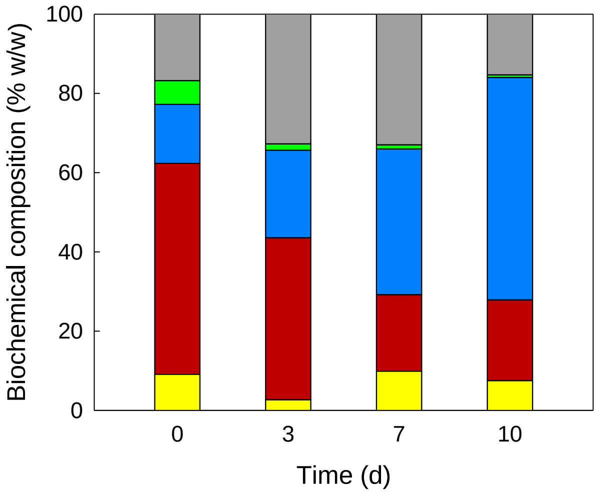


Figure 7.21. Evolution of the major biochemical components of foam-based *Chlorella sorokiniana* cultures, batch-cultivated in the liquid foam-bed photobioreactor in nitrogen-free culture medium. Major biochemical components: fatty acids (yellow), proteins (red), carbohydrates (blue), chlorophylls and carotenoids (green) and unknown compounds (grey). The surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown.

Contrary to nitrogen-starved liquid cultures, no net biomass was produced in the nitrogen-starved foam-based cultures (**Fig. 7.20A**). Hence, the productivity of carbohydrates was zero (**Table 7.5**). The rapid increased in the carbohydrate fraction of the biomass can therefore be explained by the balanced carbohydrate production and protein losses.

Furthermore, the changes in the fatty acids profile were similar to those observed in nitrogen-starved liquid cultures (**Fig. 7.22**). Although the lipid fraction of the biomass remained unchanged, the relative abundance of saturated fatty acids was again found to increase up to approximately 40% and the MUFA/PUFA ratio decreased almost by half in 7 days.

Overall, and considering the effects observed on the algal growth, photosynthetic efficiency and biochemical composition, the results suggest a more intense stress effect of nitrogen deprivation on *C. sorokiniana* cultures when this microalga is cultivated in foam.

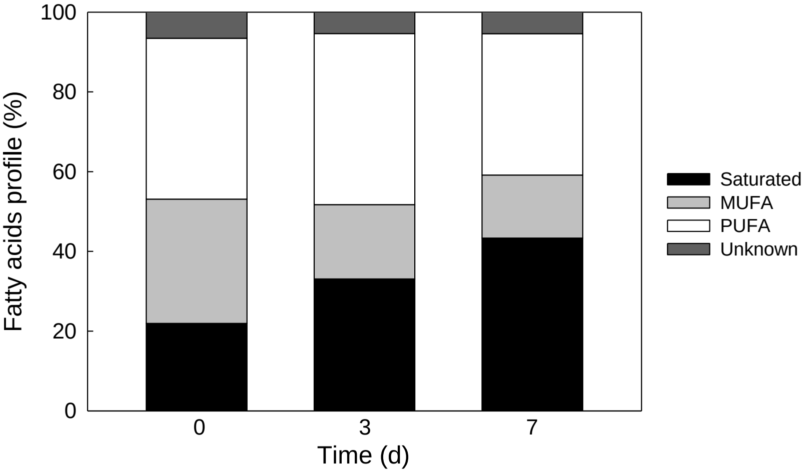


Figure 7.22. Fatty acid composition of *Chlorella sorokiniana* in nitrogen-starved foam-based cultures in the liquid foam-bed photobioreactor (LF-PBR). The fatty acid composition is expressed as the percentage of saturated, monounsaturated (MUFA), polyunsaturated (PUFA) and unknown fatty acids. The surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown.

In order to test how the microalgal cultures perform in the LF-PBR under other conditions, *C. sorokiniana* was cultivated in nutrient-replete foam-based cultures under saturating light intensities. For that, a cylindrical LED-system that provided a more homogeneous illumination and that allowed the regulation of the light intensity (Chapter 6) was used to illuminate each LF-PBR unit (**Fig. 7. 23**). The light intensity received at the LF-PBR surface (Chapter 6) was sequentially increased as follows: 157 µmol photons m^{-2} s^{-1} for the first 24 h, 239 µmol photons m^{-2} s^{-1} between 48 h and 72 h of cultivation and 431 µmol photons m^{-2} s^{-1} from 72 h of cultivation onwards. The light intensities employed can be considered saturating for low density cultures of *C. sorokiniana* but not high enough as to address photoinhibition of the photosynthesis (Cuaresma et al., 2009).

Figure 7.23. Liquid foam-bed photobioreactors illuminated by LED-systems (left, saturating light) and by the fluorescent lamps panel (right, no saturating light).

Overall, no significant differences were observed between foam-based cultures of *C. sorokiniana* when illuminated with saturating light intensity, in terms of growth and biochemical composition of the biomass (**Fig. 7.24**), when compared to those illuminated with no saturating light by the fluorescent light panel (**Fig. 7.17**). The evolution of the major biochemical components followed similar patterns (i.e. carbohydrate to protein ratio increase), although the changes were attenuated compared to those observed under no saturating light.

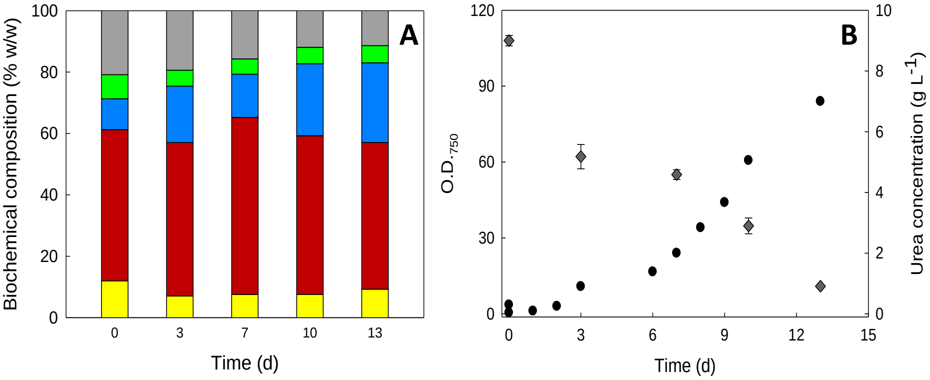


Figure 7.24. Foam-based cultures of *Chlorella sorokiniana* batch-cultivated in the liquid foam-bed photobioreactor in 3-fold concentrated M-8 culture medium under saturating light intensity. (A) Evolution of the major biochemical composition: fatty acids (yellow), proteins (red), carbohydrates (blue), chlorophylls and carotenoids (green) and unknown compounds (grey). (B) Time-course evolution of the biomass concentration in terms of optical density at 750 nm (black dots), and urea concentration (grey rhombus). The surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

However, temperature-dependent foamability of Pluronic F68 was a side result of these experiments (Chapter 6). The foamability of the cultures was at least partially lost overnight, therefore the cultures could not be maintained as foam-based cultures and were partially in liquid phase part of each day. Thus, the results obtained correspond to cultures intermittently exposed to saturating light.

Contrary to the foam-based cultures exposed to the illumination of the fluorescent lights panel (**Fig. 7.19** and **7.22**), there were no changes in the fatty acids profile of the biomass (**Fig. 7.25**). It can be noted that the fatty acids profile at the end of cultivation was an intermediate result of that observed in cultures continuously maintained in liquid and in foam (**Table 7.6**). This might have been a consequence of the discontinuity of exposure to the foam environment and the saturating light illumination.

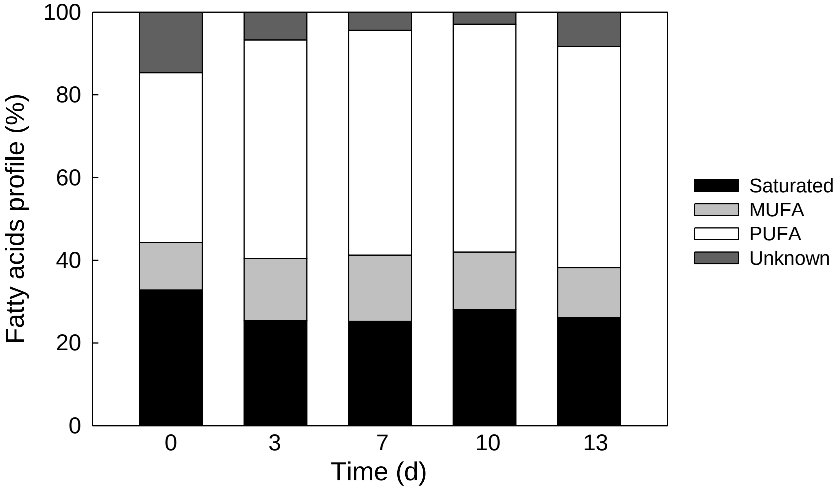


Figure 7.25. Fatty acid composition of *Chlorella sorokiniana* in foam-based cultures in 3-fold concentrated M-8 culture medium in the liquid foam-bed photobioreactor. The fatty acid composition is expressed as the percentage of saturated, monounsaturated (MUFA), polyunsaturated (PUFA) and unknown fatty acids. The surfactant Pluronic F68 was added to the foam-based cultures at the concentration of 12.5 CMC. The average values of duplicate cultures are shown.

The evolution of the chlorophylls and carotenoids cell content of the foam-based cultures is shown in **Figure 7.26**. As previously explained, the chlorophyll cell content decreased drastically when nitrogen was depleted (**Fig. 7.26A**), whereas it slightly decreased as nitrogen was being consumed in cultures grown in 3-fold culture media.

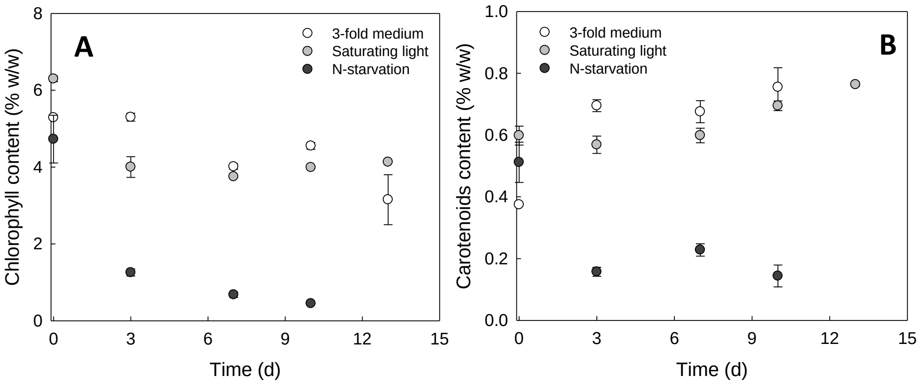


Figure 7.26. Cellular content (% w/w) of (A) total chlorophylls and (B) carotenoids of *Chlorella sorokiniana* cultivated in foam-based cultures in the liquid foam-bed photobioreactor. Culture conditions: 3-fold concentrated M-8 culture medium (3-fold medium), 3-fold concentrated M-8 culture medium under saturating light intensity (Saturating light) and nitrogen-starved cultures (N-starvation). The surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

On the other hand, the carotenoids cell content increased in cultures in 3-fold culture media regardless of the illumination provided, whereas it decreased in nitrogen-starved cultures (**Fig. 7.26B**). The potential of the species *C. sorokiniana* as a lutein producer has already been acknowledged due to its high lutein content and its fast growth (Chen et al., 2015; Cordero et al., 2011). Lutein is one the major carotenoids with commercial value that can be found in microalgae (Gong and Bassi, 2016). Among other applications, lutein is used as nutraceutical against macular degeneration. Therefore, the lutein content of *C. sorokiniana* was measured in liquid and foam-based cultures in flasks and in the LF-PBR under the different conditions assessed.

Lutein content of the cultures at the beginning of the experiment and after 3 and 10 days of cultivation are shown in **Figure 7.27**. Nitrogen starvation resulted in a deceased of lutein content in both flasks (**Fig. 7.10**) and the LF-PBR (**Fig. 7.27**). However, the cultivation of *C. sorokiniana* in foam, in a nutrient replete scenario (3-fold concentrated medium, after 10 days of cultivation, when urea was still available) resulted in a large increase of this compound. A lutein content of 5.5 mg g^{-1} was achieved in the LF-PBR after 10 days of cultivation in nutrient-replete medium. This value is similar to those obtained for *C. sorokiniana* and other lutein-rich microalgae after optimizing the cultivation conditions or by genetically improving the strains (Cordero et al., 2011; Vaquero et al., 2013). On the other hand, cultures exposed to saturating light intensity showed intermediate values, which may be explained by the intermittent exposure of the culture to the foam conditions, since these cultures performed part of the time as liquid cultures. Thus, the LF-PBR is not only a promising strategy for low-cost biomass production but also promising for the production of algal specialties such as lutein.

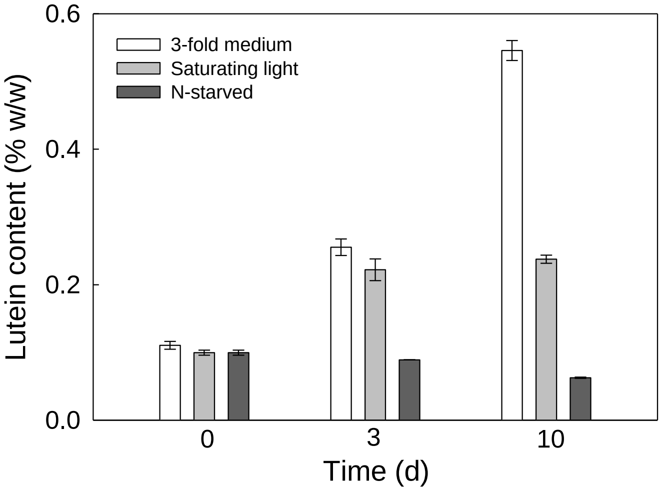


Figure 7.27. Cellular content (% w/w) of lutein of *Chlorella sorokiniana* cultivated in foam-based cultures in the liquid foam-bed photobioreactor. Culture conditions: 3-fold concentrated M-8 culture medium (3-fold medium), 3-fold concentrated M-8 culture medium under saturating light intensity (Saturating light) and nitrogen-starved cultures (N-starved). The surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

Lastly, the performance of *C. sorokiniana* foam-based cultures was assessed in a mixed scenario that consisted in nitrogen free culture medium combined with saturating light intensity. Under such conditions the foam-based cultures did not survive for more than 48h and collapsed. This suggests a higher physiological weakness of *C. sorokiniana* when cultivated in foam and subjected to such stress synergy, compared to liquid cultures under the same conditions.

7.4 Conclusions

When cultivated in the LF-PBR, *C. sorokiniana* showed a much faster growth in foam-based cultures, leading to a fast consumption of nutrients. This enhanced growth in foam might be due to: (i) physicochemical characteristics of the foam-based cultivation (e.g. high CO_2 availability), and/or (ii) possible interactions between the surfactant and the algal cell cover which might take place in the foam and improve algal growth.

However, these interactions between Pluronic F68 and *C. sorokiniana* cells were not perceptible in liquid cultures. No effects of Pluronic F68 on the algal growth or the biochemical composition of the biomass were found when added to nutrient-replete or nitrogen-starved liquid cultures of *C. sorokiniana.* Nevertheless, some interactions must take place as the surface hydrophobicity of four algal strains was modified by the presence of this surfactant. For a better understanding of the surfactant-algal cells interactions, further research should be conducted.

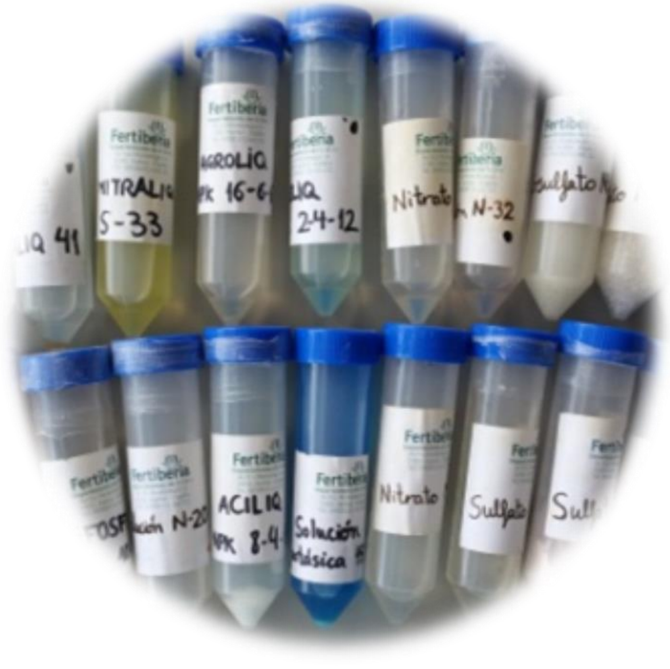
C. sorokiniana biomass produced in the LF-PBR showed to have a high carbohydrate to protein ratio, while the fatty acid content of the biomass remained unchanged along the cultivation period. However, the fatty acid profile showed in general a lower degree of unsaturation compared to liquid cultures. Under certain conditions, the cultivation in the LF-PBR showed to increase the stress response in *C. sorokiniana* (further photosynthetic efficiency decrease, cease of growth and biochemical changes). When subjected to nitrogen starvation, *C. sorokiniana* showed to rapidly accumulate carbohydrates (50% content of the biomass). Nitrogen-starvation combined with higher light intensities resulted in the collapse of the cultures when performed in foam, restating the idea that the stress effects are intensified in the LF-PBR.

Our results indicate that, beyond the surfactant effect, the “foam environment” has an effect on the metabolism of *C. sorokiniana*. Besides the changes observed in the major biochemical composition, high lutein content (5.5 mg g^{-1}) was achieved when *C. sorokiniana* was cultivated in the LF-PBR in nutrient-replete medium.

The high growth rates and biochemical composition of the biomass cultivated in the LF-PBR suggest a high potential of this cultivation system for cost-effective microalgal biomass production with distinct metabolites profile.

Chapter 8

Feasibility of fertilizer-based culture medium for microalgae cultivation in surfactant-stabilized foam



This Chapter will be submitted as:

Vázquez M, Bermejo E, González C, Vílchez C, Cuaresma M. Potential of fertilizers-based culture medium for low cost microalgae cultivation in surfactant-stabilized foam

8.1 Abstract

Cost savings derived from cultivation in surfactant-stabilized foam could be enlarged by replacing the culture media based on pure chemicals by agricultural fertilizers. NPK fertilizers are agricultural products containing, at least, nitrogen, phosphorus and potassium in a certain ratio. Besides, in a NPK fertilizer nitrogen can be provided in several forms. Therefore, in order to select an appropriate NPK fertilizer for the cultivation of a given microalgal species, both the nitrogen source and the ratio among the major nutrients supplied must be assessed. In addition, the NPK concentration should also be adequate to allow stable and productive microalgal growth.

In this Chapter, the most suitable nitrogen source for *Chlorella sorokiniana* was selected by testing 10 nitrogen fertilizers (NF). The nitrogen fertilizers evaluated were composed of nitrate, ammonium, urea and mixtures of them. Besides, the growth of this microalga was tested using 2 commercial NPK fertilizers containing the nitrogen source previously selected as the optimal one for *C. sorokiniana*. In this sense, the effects of the varying NPK ratios and the fertilizer concentration on algal growth and on the pH of the algal culture were evaluated. Moreover, the adequacy of a Pluronic F68-added NPK-based culture medium was assessed in liquid cultures of *C. sorokiniana*. According to the biomass and lutein productivities obtained, as well as the potential cost savings, the results indicate that the usage of agricultural fertilizers can be a promising tool to achieve economically sustainable microalgal production in foam.

8.2 Introduction

Microalgae production in surfactant-stabilized foam has been established as a promising alternative to conventional cultivation methods in order to make microalgae production processes economically feasible. In this sense, several designs for liquid foam-bed photobioreactors (LF-PBR) have been proposed (Chapter 6; Janoska et al., 2017, 2018a). Regardless of the design, this innovative cultivation concept offers several advantages that allow cultivation at high biomass densities, which may lead to reduced water consumption during cultivation and also to lower harvesting costs (Janoska et al., 2017; 2018a).

Besides the advantages of cultivating microalgae in a LF-PBR, other aspects of the cultivation process should be tackled in order to maximize the potential of this cultivation concept at large scale. In this sense, the availability of suitable and cheap nutrients sources is one of the key aspects for making microalgae cultivation efficient and economically feasible at large scale (Lam and Lee, 2012; Nayak et al., 2016). In this regard, several alternatives to refined and expensive chemicals have been investigated as culture media for microalgae at large scale, including wastewater (Cai et al., 2013), manure (Hu et al., 2012) and urine (Tuantet et al., 2014). Besides, different attempts have been done where commercial fertilizers have been proved to be feasible for microalgae cultivation (Ammar, 2016; Camacho-Rodríguez et al., 2013; Nayak et al., 2016; Scardoelli-Truzzi and Sipaúba-Tavares, 2017). Thus, replacement of chemicals by agricultural fertilizers is proposed as an approach to make microalgae cultivation easier and cheaper.

The use of commercially available fertilizers is convenient since they can provide most of the major nutrients required for microalgal growth, avoiding the time-consuming media preparation based on expensive refined chemicals. NPK fertilizers simultaneously provide nitrogen, phosphorus and potassium, which are three of the major nutrients required for algal growth (Forján et al., 2015). Nitrogen is considered the most important element after carbon, since it is fundamental for the synthesis of nucleic acids and proteins (Juneja et al., 2013). Phosphorus is essential for many biochemical processes, including energy transfer reactions and, similarly to nitrogen, it is also required for nucleic acid biosynthesis (Forján et al., 2015). Finally, potassium is also fundamental in osmoregulation and it is involved in metabolic functions (Devi et al., 2013). Next to the aforementioned macronutrients, other elements such as sulfur and trace metals are required for the normal growth and metabolic functioning of microalgae (Forján et al., 2015; Juneja et al., 2013). Therefore, in addition to using a suitable NPK, fertilizer-based culture media can be supplemented with an agricultural micronutrients solution in order to provide a complete combination of all the elements required for microalgal growth.

In general, a NPK fertilizer provides phosphorus and potassium in chemical forms suitable for plant usage (orthophosphate ion and K^+). However, in a NPK fertilizer nitrogen can be provided in several chemical forms, including compounds based on ammoniacal nitrogen, nitrate, ureic nitrogen, or a combination of all of them. Thus, prior to select a NPK fertilizer that is optimal for cultivating a specific microalga, the nitrogen source present in the NPK fertilizer must be assessed in terms of suitability for microalgal growth. To do this, the nitrogen source of a reference culture medium can be replaced by nitrogen fertilizers (NF), which are N-products commercially available that resemble the nitrogen sources that can be contained in NPK fertilizers.

As explained above, cultivation of microalgae in foam was evidenced to be an innovative biomass production process alternative in which high productivities and high energy savings can be achieved compared to algal cultivation in conventional photobioreactors. However, previous results also demonstrated that cultivation of microalgae in foam requires the presence of an appropriate surfactant which stabilizes the formed foam (Chapter 3; Vázquez et al., 2018). In this regard, Pluronic F68 was selected as a promising surfactant for microalgae cultivation in foam due to the following characteristics: (i) it owns good foaming properties, (ii) it holds the microalgae up in the foam formed, (iii) it is not rapidly biodegradable, and (iv) it is non-toxic for microalgae (Chapter 4; Janoska and Vázquez et al., 2018). The use of Pluronic F68 for the growth of the microalga *Chlorella sorokiniana* in surfactant-stabilized foam was then proved in different LF-PBR systems (Chapter 7; Janoska et al., 2018a). So far, microalgal growth in Pluronic F68-stabilized foam has been achieved for *Chlorella sorokiniana* (Chapter 7; Janoska et al., 2018a) and *Chlorella vulgaris* (Janoska et al., 2018a). Therefore, Pluronic F68-added *C. sorokiniana* cultures would be suitable to assess the adequacy of fertilizers-based algal culture media in order to reduce foam-based biomass production costs further. In this sense, parameters such as microalgal growth, as well as the content of its commercially relevant compounds (i.e. lutein in the case of *C. sorokiniana*) can be assessed to determine whether a given fertilizer-surfactant combination could be suitable for algal cultivation in foam.

According to the stated above, this Chapter focuses on: (i) the selection of an optimal nitrogen source for *Chlorella sorokiniana* growth by testing 10 agricultural nitrogen fertilizers (NF), including products based on ammonium, nitrate, urea, and mixed nitrogen sources; (ii) the assessment of two NPK fertilizers with varying NPK ratios, as well as the effect of different NPK concentrations on *C. sorokiniana* growth and the stability of the culture pH, and (iii) the study of the compatibility between an agricultural fertilizer (i.e. NPK) and the Pluronic F68 surfactant. For the latter, the growth of *C. sorokiniana* and the productivity of photosynthetic pigments were evaluated in Pluronic F68-added NPK-based liquid cultures and compared with the results obtained in standard cultures of *C. sorokiniana* grown in a reference culture medium.

8.3 Results and discussion

8.3.1 The effect of nitrogen fertilizers on *Chlorella sorokiniana* growth

As a first approach to replace the culture medium of *Chlorella sorokiniana* by an agricultural NPK fertilizer, a screening of optimal nitrogen sources for the growth of this microalga was performed. *C. sorokiniana* was cultivated in 24-well plates using modified M-8 culture medium, in which the original nitrogen source of the medium (i.e. KNO_3) was replaced by 10 different nitrogen fertilizers (NF). The NFs were added to nitrogen-free M-8 media at the corresponding concentration in order to approximate the original nitrogen concentration of the medium, 0.03 M. The NFs tested covered the most common nitrogen sources used by microalgae: nitrate, ammonium and urea (Forján et al., 2015), as well as mixtures of them. The agricultural fertilizers employed and their compositions are listed in **Table 2.2**.

Figure 8.1 shows the growth rates of the different cultures after 72 hours of cultivation. The growth rates were calculated using the optical density values at 680 nm, and the values were normalized according to the initial optical density of each culture.

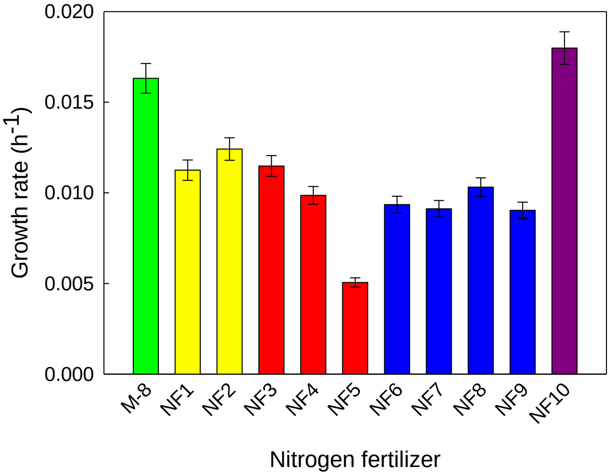


Figure 8.1. Growth rate, expressed as normalized O.D.$_{680}$ rate (h^{-1}), of *Chlorella sorokiniana* after 72 hours of cultivation in 24-well plates in the different media. Control culture (green bar): M-8 culture medium (M-8). Fertilizers based on nitrate (yellow bars): potassium nitrate (NF1) and magnesium nitrate (NF2). Fertilizers based on ammonium (red bars): ammonium sulfate (NF3), ammonium thiosulfate (NF4) and ammonium polyphosphate (NF5). Fertilizers based on mixed nitrogen sources (blue bars): N-20 solution (NF6), N-32 solution (NF7), Nitraliq S-33 (NF8) and Sulfaliq 41 (NF9). Liquid urea (purple bar) (NF10). The average values of dulicate cultures are shown, together with their corresponding standard deviations. The initial nitrogen concentration was 0.03 M in all cases.

As observed in **Figure 8.1**, all cultures containing the nitrogen fertilizers grew slower than the control culture (M-8) during the first 72 hours, except for that with agricultural urea solution (NF10), which showed a growth rate similar to that of the control culture. Regarding the different NFs employed, cultures with only nitrate as nitrogen source (NF1 and NF2) showed a slightly better performance than those with only ammonium (NF3, NF4 and NF5) and those with mixed nitrogen sources (NF6-9). In particular, cultures with ammonium polyphosphate (NF5) showed the lowest growth rate, which was more than 3-fold lower than those of control cultures (M-8) and cultures with urea solution (NF10). In general, fertilizers solely based on nitrate or urea showed better performance than those solely based on ammonium or containing ammonium.

According to the preliminary results obtained in microplates, the nitrogen fertilizers based on nitrate (NF1 and NF2), those based on mixed nitrogen sources containing urea (NF7, NF8, and NF9) and the agricultural urea solution (NF10) were further studied as potential nitrogen sources for *C. sorokiniana*. The microalga was then cultivated in batch mode in flasks for 9 days in modified M-8 culture medium, in which the original nitrogen source of the medium was replaced by the selected NFs. The NFs were added to nitrogen-free M-8 media at the corresponding concentration in order to approximate the original nitrogen concentration of the medium, 0.03 M. In this assay, two control cultures were included, which contained either urea or potassium nitrate as nitrogen source. **Table 8.1** enlists the maximal optical density at 680 nm and the maximal growth rate achieved by the different cultures. The growth rates were calculated for the period in which linear growth was observed (i.e. first 163 hours of cultivation).

Table 8.1. Maximal optical density at 680 nm and maximal growth rate (d^{-1}) achieved by *Chlorella sorokiniana* in batch cultures in modified M-8 culture medium containing nitrogen fertilizers (NFs): fertilizers based on nitrate (NF1 and NF2), fertilizers based on mixed nitrogen sources containing urea (NF7, NF8 and NF9) and agricultural urea solution (NF10). Cultures in M-8 culture medium containing urea and potassium nitrate (refined chemicals) were used as controls. Maximal growth rate is expressed as maximal $O.D._{680}$ rate (d^{-1}). In all cases the initial $O.D._{680}$ of the cultures was 0.25 and the initial nitrogen concentration was 0.03 M. The average values of dupliate cultures are shown, together with their corresponding standard deviations.

	Nitrogen compound (% w/w)			**Maximal $O.D._{680}$**	**Maximal growth rate (d^{-1})**
	Nitrate	**Ammonium**	**Urea**		
Control KNO_3	100	-	-	4.79 ± 0.06	0.69 ± 0.07
Control Urea	-	-	100	8.81 ± 0.15	1.02 ± 0.02
NF1	100	-	-	4.12 ± 0.20	0.59 ± 0.03
NF2	100	-	-	3.01 ± 0.09	0.44 ± 0.11
NF7	25	25	50	4.13 ± 0.07	0.59 ± 0.09
NF8	11.15	20.77	68.08	6.67 ± 0.21	0.87 ± 0.02
NF9	22.86	31.43	45.72	4.52 ± 0.18	0.62 ± 0.01
NF10	-	-	100	8.20 ± 0.22	1.02 ± 0.02

It can be observed in **Table 8.1** that both growth parameters were maximal when urea was used as nitrogen source, either added as a pure chemical (Control Urea) or as an agricultural urea solution (NF10). Among the other NF, the fertilizers based on mixed nitrogen sources containing urea (NF7, NF8 and NF9) performed better than those based on nitrate (NF1 and NF2). In particular, cultures with the fertilizer NF8 achieved higher biomass densities and grew faster than the rest. It should be noted that this fertilizer contained the highest proportion of ureic nitrogen among the products based on mixed nitrogen sources (**Table 8.1**).

Considering all cultures were prepared at the same initial nitrogen concentration and since enhanced growth was observed in cultures with urea as the only nitrogen source, a possible correlation between the percentage of nitrogen added in form of urea and *C. sorokiniana* growth was hypothesized. **Figure 8.2** shows the maximal growth rates (**Fig. 8.2A**) and the maximal O.D.$_{680}$ values (**Fig. 8.2B**) achieved by *C. sorokiniana* cultures in modified M-8 culture medium with nitrogen fertilizers containing urea as a part of the nitrogen composition (NF7, NF8 and NF9) and as the sole nitrogen source (NF10). Besides, the growth rates and the maximal O.D.$_{680}$ values of the control culture containing urea reagent as nitrogen source are also shown. All the data are presented in relation to the percentage of nitrogen in urea form contained in the fertilizer (X axis).

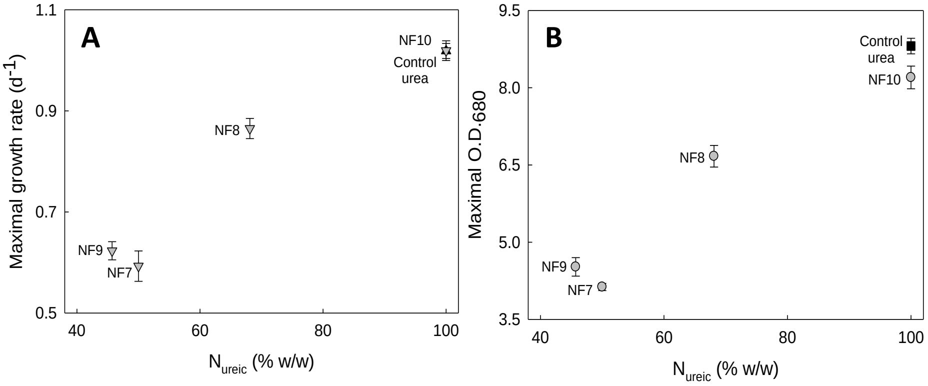


Figure 8.2. Correlation between the ureic nitrogen content of the culture medium (% w/w) and (A) the maximal growth rate, expressed as maximal O.D.$_{680}$ rate (d^{-1}), and (B) the maximal biomass density, expressed as maximal O.D.$_{680}$ values, achieved by *Chlorella sorokiniana. C. sorokiniana* was cultivated in batch cultures in modified M-8 culture medium containing nitrogen fertilizers (NFs): fertilizers based on mixed nitrogen sources containing urea (NF7, NF8 and NF9) and agricultural urea solution (NF10) (grey symbols). Algal cultures in M-8 culture medium containing urea (refined chemicals) were used as controls (black symbols). In all cases the initial O.D.$_{680}$ of the cultures was 0.25 and the initial nitrogen concentration was 0.03 M. The average values of dupliate cultures are shown, together with their corresponding standard deviations.

It can be inferred from **Figure 8.2** that there is a positive correlation between the content of ureic nitrogen and *C. sorokiniana* growth. This restates that urea is the most optimal nitrogen source for the cultivation of this microalga, among those investigated in this study. Besides, there were no large differences between the growth of cultures with urea reagent and that with agricultural urea solution, which indicates the potential benefit of using agricultural urea solution as nitrogen source for *C. sorokiniana* cultivation.

For cultivating many microalgal species, ammonium is preferred to nitrate as nitrogen source since the consumption of ammonium is energetically advantageous if compared to that of nitrate. Nitrate has to be reduced to nitrite and then to ammonium before it can be finally incorporated into carbon skeletons for aminoacids biosynthesis (Vega, 2018). In the particular case of *C. sorokiniana*, this preference for ammonium over nitrate has been already reported (Lizzul et al., 2014). However, our results show that urea promotes *C. sorokiniana* growth further than nitrate and ammonium, which is in line with previous observations for this microalga (Ramanna et al., 2014) and other algal species such as *Chlorella protothecoides* (Shi et al., 2000), *Coccomyxa onubensis* (Casal et al., 2011) or *Nannochloropsis salina* (Campos et al., 2014). Urea ($CO(NH_2)_2$) is a small molecule that dissociates into ammonium and bicarbonate and can, therefore, be considered as a combined source of nitrogen and carbon (Casal et al., 2011). It is known that, in microalgae, two enzymes can metabolize urea: urease (EC: 3.5.1.5) and urea amidolyase (UALse) (EC: 6.3.4.6) (Pérez-García et al., 2011). However, most *Chlorella* spp. apparently lack urease and metabolize urea by UALse (Syrett and Leftley, 2016), which requires ATP for its activity:

$$\mathbf{CO(NH_2)_2 + ATP + H_2O \rightarrow CO_2 + 2NH_3 + ADP + Pi}$$

The results presented above correspond to *C. sorokiniana* cultures performed in microplates and flasks, which were supplied with CO_2-enriched air either by bubbling it directly into the cultures (flasks) or by maintaining a CO_2-enriched atmosphere in the cultivation space (microplates). Nonetheless, the extra CO_2 supplied by urea might have contributed to the greater growth observed with this nitrogen source compared to that with fertilizers based on nitrate and/or ammonium.

8.3.2 Selection of a suitable NPK agricultural fertilizer for the cultivation of *Chlorella sorokiniana*

Once urea was determined as the most optimal nitrogen source for *C. sorokiniana*, 2 agricultural fertilizers (NPK) containing urea as the main nitrogen source were tested in order to study the potential of NPK-based culture media for the cultivation of this microalga. The NPK used contained different nitrogen, phosphorous and potassium ratios: NPK 18-6-6 and NPK 12-6-4, resulting in molar nitrogen to phosphorous ratios (N/P) of 13.5 and 10, respectively (see Materials and Methods, **Section 2.2.3**). In terms of molar concentration, urea represented 88.6% and 76.5% of the total nitrogen in NPK 18-6-6 and NPK 12-4-6, respectively; the remaining percentage being in form of ammoniacal nitrogen.

Since tap water would be more convenient and cheaper than distilled water for algal culture media preparation, the suitability of tap water for NPK-based culture media was assessed. For this, *C. sorokiniana* was cultivated in batch cultures in 24-well plates containing 1 mL of NPK (i.e NPK 18-6-6 or NPK 12-6-4) per liter of tap or distilled water for 3 days. The maximal optical densities achieved by the cultures are presented in **Figure 8.3.**

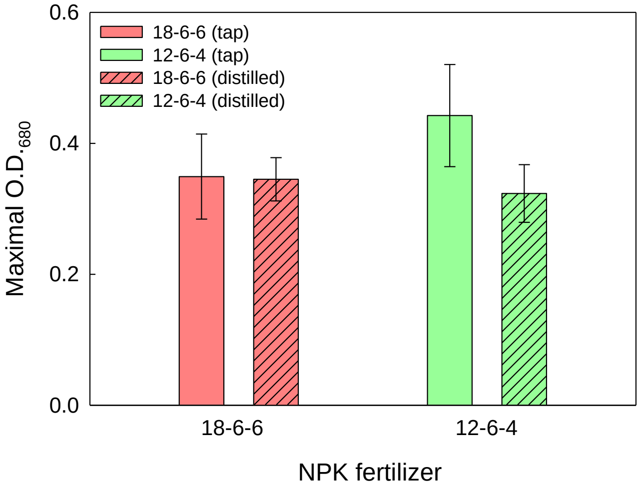


Figure 8.3. Maximal optical density at 680 nm (maximal O.D.$_{680}$) of *Chlorella sorokiniana* batch cultures in 24-well plates. Culture media: NPK 18-6-6 and NPK 12-6-4. NPK-based culture media were prepared at the concentration of 1 mL of NPK per liter of in tap water or distilled water. The initial O.D.$_{680}$ of the cultures was 0.15 in all cases. The average values of duplicate cultures are shown, together with their corresponding standard deviations.

As can be observed, both NPK allowed microalgal growth. Moreover, no large differences were observed among cultures with NPK prepared in tap or distilled water. In fact, cultures in NPK-based media prepared in tap water performed slightly better than those in distilled water, possibly due to the presence of certain elements (e.g. Ca, Mg), although this was not further investigated. These results indicate the suitability of tap water for algal culture media preparation using these NPK fertilizers.

In order to get a better insight into the performance of these NPK-based culture media in microalgal growth, both NPK fertilizers based on urea were further tested in repeated-batch cultures performed in flasks at concentrations of 1 and 5 mL L^{-1}. Cultures at 5 mL L^{-1} would allow to identify possible nutrients limitations that may occur at 1 mL L^{-1}. The NPK-based culture media were prepared in tap water, since it showed to perform slightly better than distilled water (**Fig. 8.3**). Besides, the usage of tap water would become advantageous for the application of NPK-based culture media at large scale, since it would make the media preparation process easier and cheaper compared to the use of distilled water. The cultures were maintained within the optical density range of 1.2-2.5 for a time period of 100 hours (**Fig. 8.4A**), since batch cultures of *C. sorokiniana* previously performed had shown to grow linearly in this range of optical density values (data not shown). In order to avoid growth limitations due to lack of trace elements, the NPK-based culture media were supplemented with 0.1 mL L^{-1} of the agricultural micronutrients solution described in Materials and Method, (**Section 2.2.3**), as well as with 0.4 g L^{-1} of $MgSO_4$, since the micronutrients solution did not contain sulfur and this element is one of the macronutrients required for microalgae production (Forján et al., 2015).

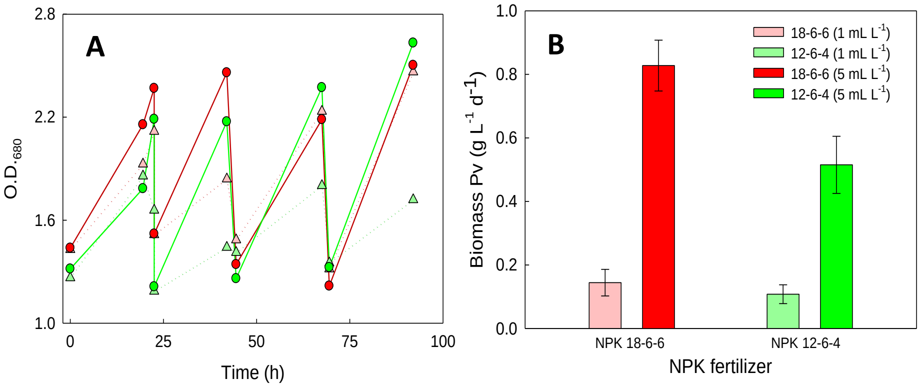


Figure 8.4. (A) Optical density (O.D.$_{680}$) evolution of repeated-batch cultures of *Chlorella sorokiniana* in NPK-based culture media: NPK 18-6-6 (red symbols) and NPK 12-6-4 (green symbols) at concentrations of 1 mL (dotted lines, triangles) and 5 mL (solid lines, circles) of NPK per liter of culture broth. (B) Volumetric biomass productivity of *Chlorella sorokiniana* (Biomass P_v), expressed as grams of biomass produced per liter of culture and per day in repeated-batch cultures in NPK-based culture media: NPK 18-6-6 and NPK 12-6-4 at concentrations of 1 mL and 5 mL of NPK per liter of culture broth. The values represent the biomass volumetric productivities accumulated during the four cycles of growth along the repeated-batch cultivation. The average values of duplicate cultures are shown, together with their corresponding standard deviations.

As observed in **Figure 8.4A**, *C. sorokiniana* grew in all the NPK-based media tested, and the algal growth was similar for both NPK added at the same concentrations (1 or 5 mL L^{-1}). However, differences were found between cultures added with 1 and 5 mL of fertilizer per liter of culture, with a higher growth observed at the highest NPK concentration regardless of the NPK added.

The volumetric biomass productivities of the cultures were also calculated for each growing cycle and results are presented in **Figure 8.4B**. The figure shows the volumetric biomass productivities accumulated during the four cycles of growth. It can be observed that the culture media based on both NPK tested allowed higher biomass productivities when the fertilizer was added at 5 mL L^{-1}, compared to those at 1 mL L^{-1}. The highest biomass productivities achieved with 5 mL per liter of the NPK 18-6-6 might be explained by two facts: (i) a more suitable ratio in which nutrients are provided, and/or (ii) a higher total nitrogen content provided. Besides, the fact that lower growth was achieved with NPK 12-6-4 might also indicate that the nitrogen provided at 5 mL of NPK per liter of this fertilizer could still be at a limiting concentration for the growth of this microalga.

Since NPK 18-6-6 resulted as a promising fertilizer, it was decided to assess its possible toxicity over *C. sorokiniana* growth at concentrations up to 20 mL L^{-1}. *C.*

sorokiniana was cultivated in batch mode at 1, 3, 5, 10 and 20 mL of NPK per liter of culture. As previously done, cultures were supplemented with 0.1 mL L^{-1} of micronutrients solutions as well as with 0.4 g L^{-1} of $MgSO_4$.

Figure 8.5A shows the volumetric biomass productivity achieved by the cultures after 3.5 days of cultivation. In general, no differences were found among the productivities of the cultures, except for the culture with the lowest concentration of NPK 18-6-6, which showed a slightly lower productivity. Moreover, the biomass yield of the NPK ($Y_{X/N}$) was also calculated. This parameter refers to the dry weight of biomass produced per mL of NPK added and the values obtained after 3.5 days of cultivation are shown in **Figure 8.5B**. It can be inferred from the figure that there is an inversely proportional relationship between the biomass yield of the NPK and its concentration. The maximal biomass yield was achieved with the lowest NPK concentration tested (i.e. 1 mL L^{-1}), which indicates that, under the experimental conditions tested, even low NPK concentrations (i.e. ≤3 mL L^{-1}) allowed nutrient availability and efficient consumption by the microalgal cells, although biomass productivities were affected at the lowest NPK concentrations (**Fig. 8.5A**).

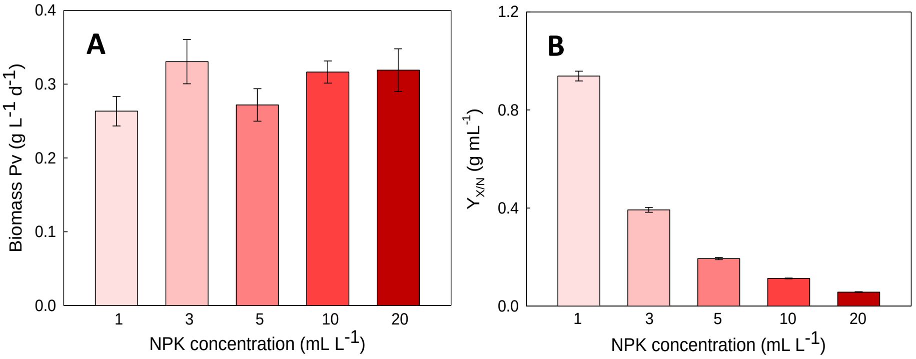


Figure 8.5. (A) Volumetric biomass productivity (Biomass P_v), expressed as grams of dry biomass produced per liter of culture and per day and (B) biomass yield, expressed as grams of dry biomass produced per mL of NPK, in batch cultures of *Chlorella sorokiniana* cultivated in NPK-based culture media for 3.5 days. NPK 18-6-6 was tested at 1, 3, 5, 10 and 20 mL of NPK per liter of culture. The average values of duplicate cultures are shown, together with their corresponding standard deviations.

On the other hand, the fact that cultures with the lowest NPK concentration (i.e. 1 mL L^{-1}) grew to a lower extent (**Fig. 8.5A**) may not have been caused, or not exclusively, by nutrients limitation, but also by the pH drop observed in these cultures. **Figure 8.6** shows the pH evolution of the cultures along the batch cultivation, during which the pH was measured daily and adjusted to 6.8 when needed. The pH of cultures with higher NPK concentration (i.e. ≥3 mL L^{-1}) slightly dropped in each growth cycle, and the pH decreases observed were not large enough to be considered an influential factor for *C. sorokiniana* growth. On the other hand, cultures with NPK added at the lowest concentration (i.e. 1 mL L^{-1}) suffered larger pH alterations. In these cultures, pH dropped to values close to 3, which is out of the optimal pH range for *C. sorokiniana* since higher specific growth rates have been observed for this microalga within the pH range between 4 and 7 (Morita et al., 2000). Therefore, these pH fluctuations observed in cultures with NPK at low concentrations possibly contributed to the lower growth observed in cultures with NPK at 1 mL L^{-1}.

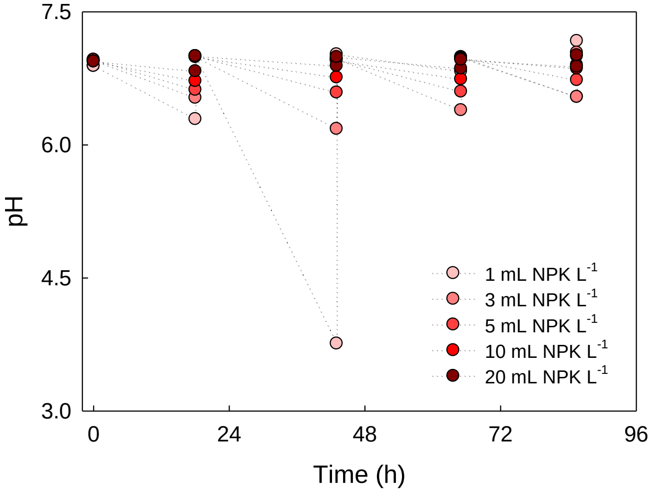


Figure 8.6. Time-course evolution of the pH of batch cultures of *Chlorella sorokiniana* in NPK-based culture media: NPK 18-6-6 at concentrations of 1, 3, 5, 10 and 20 mL of NPK per liter of culture. The average values of duplicate cultures are shown.

It can be concluded that the agricultural fertilizer NPK 18-6-6 is not toxic for *C. sorokiniana* when added at concentrations up to 20 mL L^{-1}. However, especial attention must be paid at the pH stability of the cultures when using NPK solutions at low concentrations.

Based on the previously commented results, it was hypothesized that the phosphorus provided by the NPK could be involved in buffering the pH of the cultures. Therefore, the possible role of phosphorous inorganic species in buffering the pH was assessed by following the pH of batch cultures of *C.*

sorokiniana with varying NPK and phosphoric acid concentrations. The microalga was cultivated in batch mode in culture media with 1 and 2.5 mL of NPK 18-6-6 per liter, and in cultures with 1 mL of NPK per liter but supplemented with phosphoric acid to equal the concentration of phosphorous in cultures with 2.5 mL L^{-1} of NPK. The phosphoric acid was supplied by an agricultural P_2O_5 solution 52% (w/w) and, as previously done, 0.1 mL L^{-1} of micronutrients solution and 0.4 g L^{-1} of $MgSO_4$ were added to all the conditions.

The pH evolution of the cultures is presented in **Figure 8.7**. As expected, the pH of cultures with 5 mL L^{-1} of NPK remained stable, while those of cultures with 1 mL L^{-1} of NPK dropped along cultivation. On the other hand, the pH values of cultures with 1 mL L^{-1} of NPK supplemented with phosphoric acid remained constant and similar to those of cultures with 5 mL L^{-1} of NPK. This confirmed the hypothesis that phosphorous inorganic species, particularly the species $H_2PO_4^-$ and HPO_4^{2-}, were responsible for buffering the cultures at neutral pH.

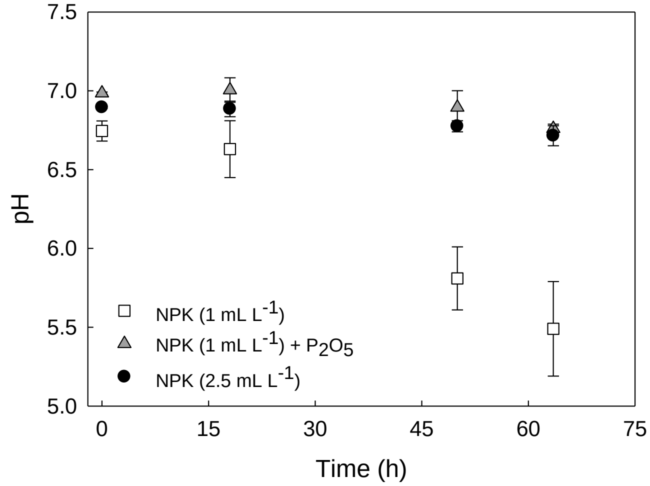


Figure 8.7. Time-course evolution of the pH of batch cultures of *Chlorella sorokiniana* in NPK-based culture media: NPK 18-6-6 at 1 mL L^{-1}, NPK 18-6-6 at 1 mL L^{-1} and supplemented with phosphoric acid solution and NPK 18-6-6 at 5 mL L^{-1}. The average values of duplicate cultures are shown, together with their corresponding standard deviations.

According to the results presented, and considering the potential application of NPK fertilizers in large scale cultures of *C. sorokiniana,* it can be concluded that the optimal NPK concentration should not be too high in order to minimize NPK waste, but too low NPK concentrations should also be avoided in order not to compromise the pH of the cultures, since the phosphorus provided by the NPK plays an important role in buffering the culture pH.

8.3.3 Feasibility of fertilizer-based culture medium for microalgae cultivation in surfactant-stabilized foam

The feasibility of NPK-based culture media with the addition of the surfactant Pluronic F68 was assessed in order to determine whether NPK fertilizers could be applied to cultivation of microalgae in a liquid foam-bed photobioreactor (LF-PBR). Pluronic F68 was used as reference surfactant since it had shown the largest potential for microalgae cultivation in foam (Chapter 4). Growth and productivity of photosynthetic pigments of *C. sorokiniana* were evaluated in NPK-based liquid cultures and compared with the results obtained in cultures grown in modified M-8 culture medium (reference cultures) (**Fig. 8.8**). Photosynthetic pigments were selected as reference metabolites to evaluate effects in the biochemical composition, due to the potential value of lutein (Bernal et al. 2011; Borowitzka, 2013), main carotenoid present in *C. sorokiniana* (Chen et al. 2015). Repeated batch cultivation of *C. sorokiniana* in flasks was carried out in order to lead to the adaptation to the new culture medium and the microalgal performance was evaluated during the non-adapted (first batch cultivation) and the adapted phase (last stage of the repeated batch cultivation). In these experiments, NPK 8-6-6 was used as fertilizer in order to adjust the nitrogen content of the medium to that of the reference medium, in which nitrogen concentration was also reduced to avoid nutrients waste. This NPK contained urea as the only nitrogen source, which was previously proved to be the most adequate for *C. sorokiniana* growth (**Section 8.3.1** of this Chapter). Since the NPK fertilizers that should be used for *C. sorokiniana* growth would contain urea and, inorganic species of phosphorous and potassium suitable for their uptake, possible incompatibilities between the NPK components and the Pluronic F68 surfactant were considered to occur regardless of the particular NPK ratio.

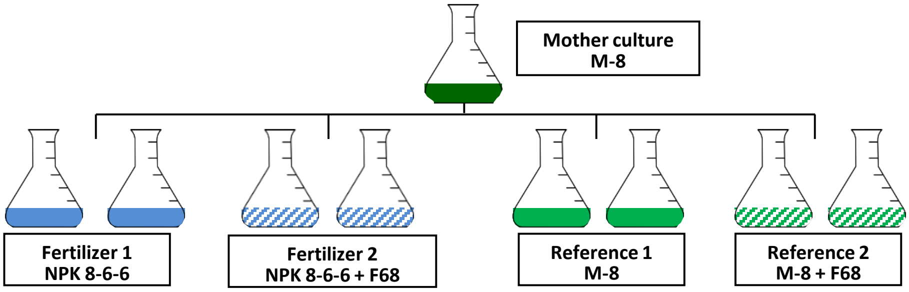


Figure 8.8. Experimental conditions evaluated during the assessment of fertilizer suitability for the cultivation of *Chlorella sorokiniana* in liquid cultures with the presence of Pluronic F68.

In order to resemble the molar composition of the reference media (M-8 and M-8 + F68), the amount of commercial fertilizer used (NPK 8-6-6) was approximated to the nitrogen content of the reference media. Considering the molar composition of the reference media is committed to high cell density cultures operated in continuous mode in photobioreactors, it was decided to reduce the amount of main nutrients to one-third in the reference media to avoid nutrients waste during the repeated-batch experiments in flasks. Besides, in order to avoid growth limitation due to the lack of trace elements in the commercial NPK, the commercial solution of micronutrients previously employed (**Section 8.3.2** of this Chapter) was also used and its concentration was adjusted according to the iron content of the reference media. Finally, and due to the use of distilled water to prepare the medium, calcium, magnesium and sulfur were also added to the NPK-based media. The addition of calcium and magnesium could be avoided in cultivation at large scale, for which tap water would be efficiently used (**Fig. 8.3**). The final composition of the different media employed, as well as the composition of the commercial fertilizer and micronutrients solution, is shown in Materials and Methods (**Sections 2.2.3** and **2.3.9**). Media supplemented with the surfactant Pluronic F68 were prepared at a concentration of 12.5 CMC (Critical Micelle Concentration = 0.334 g L^{-1}), since it showed to be optimal for the operation of our LF-PBR (Chapter 6).

During the repeated-batch cultivation the daily determination of biomass density allowed to maintain the different cultures in an optimal range (optical density at 750 nm was maintained between 0.3 and 2.5) by punctual dilutions. During 42 days of cultivation pH was maintained at 7.0 ± 0.5 and maximal photosynthetic efficiency of photosystem II (PSII) was daily monitored. Biomass dry weight and pigment content (chlorophyll and carotenoids) were determined during the first and the last growing cycle as representative from non-adapted and adapted cultures respectively.

Figure 8.9 shows the evolution of biomass concentration of the different cultures, measured as optical density at 750 nm and normalized to the initial biomass concentration of each growing cycle. As observed, the different batch cycles shortened with the periodic dilution as a result of growth adaptation. The NPK-based media showed a slightly lower biomass growth when compared to the reference media. Accordingly, in the adapted phase, a 14% and 22% average reduction was observed in cultures without surfactant and with the addition of it, respectively. It is noteworthy that the presence of the surfactant did not result in a negative effect. This is in agreement with previous results already obtained from experiments in liquid cultures, where the presence of Pluronic F68 did not alter *C. sorokiniana* growth (Chapter 7).

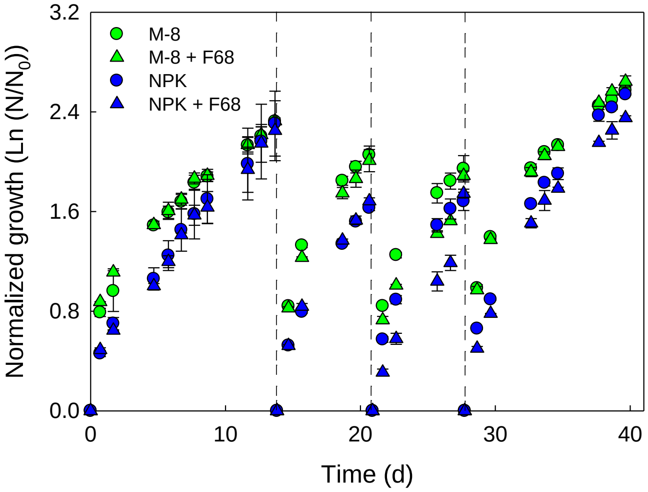


Figure 8.9. Time-course evolution of *Chlorella sorokiniana* biomass density in the different media, expressed as normalized growth (optical density at 750 nm), during the repeated-batch cultivation. Culture media: reference medium M-8 (M-8), reference medium M-8 with the addition of Pluronic F68 at 12.5 CMC (M-8 + F68), NPK 8-6-6 medium (NPK) and NPK 8-6-6 medium with the addition of Pluronic F68 at 12.5 CMC (NPK + F68). Dash lines represent the punctual dilutions carried out and error bars represent the standard deviation of duplicate cultures.

Same pattern was found for the maximal photosynthetic efficiency of Photosystem II (F_v/F_m) (**Fig. 8.10**). Minor differences were observed between the reference media and the NPK-based media and all the cultures showed an efficiency above

0.6, which is considered an optimal value for microalgae (Schreiber et al. 1995). Besides, no differences were found when the surfactant was present, neither in the reference medium nor in combination with the fertilizer, which is in line with previous results presented in this Thesis (Chapter 7).

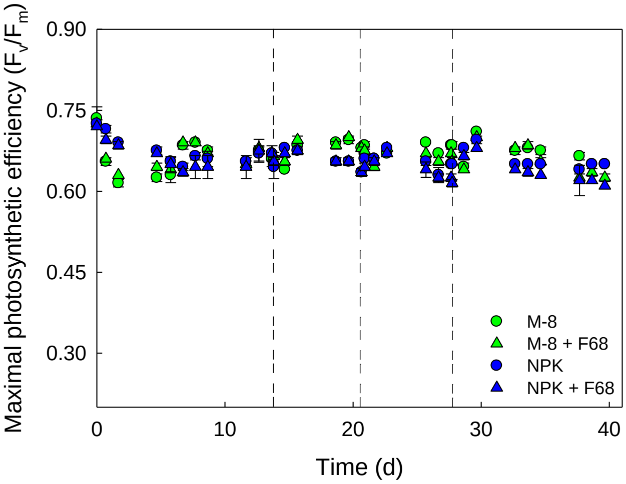


Figure 8.10. Time-course evolution of maximal photosynthetic efficiency of Photosystem II (F_v/F_m) of *Chlorella sorokiniana* during the repeated-batch cultivation. Culture media: reference medium M-8 (M-8), reference medium M-8 with the addition of Pluronic F68 at 12.5 CMC (M-8 + F68), NPK 8-6-6 medium (NPK) and NPK 8-6-6 medium with the addition of Pluronic F68 at 12.5 CMC (NPK + F68). Dash lines represent the punctual dilutions carried out and error bars represent the standard deviation of duplicate cultures.

A closer look at the non-adapted and the adapted phases confirmed the results mentioned above (**Fig. 8.11**). Biomass cell concentration, measured as biomass dry weight, was higher in the standard media than in the NPK-based culture media. These differences were slight during the non-adapted phase (7.3% average reduction in the NPK-based media) but they increased during the adapted phase (26.1% average reduction). On the other hand, only a minor difference was found when the surfactant was present, which confirmed that the surfactant Pluronic F68 did not compromise *C. sorokiniana* growth. Finally, adaptation of *C. sorokiniana* by repeated-batch cultivation resulted in a higher cell density in a shorter period of time for all the conditions assessed, which has a direct impact in the biomass productivity (**Fig. 8.12**).

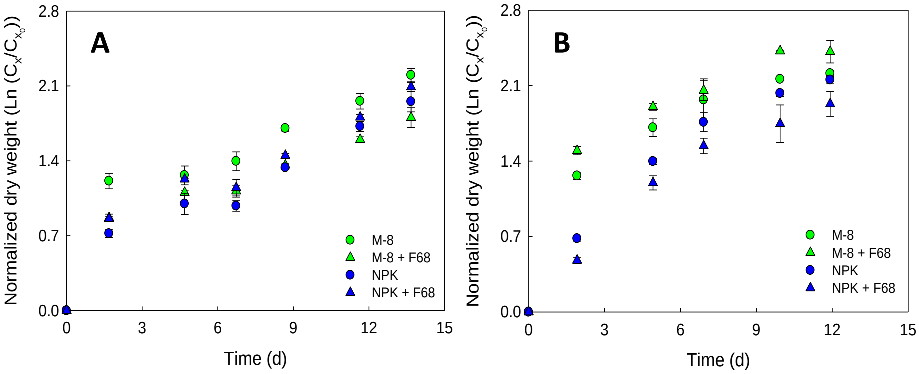


Figure 8.11. Time-course evolution of biomass dry weight of *Chlorella sorokiniana*, expressed as normalized dry weight (g L^{-1}), during (A) the non-adapted phase and (B) the adapted phase. Culture media: reference medium M-8 (M-8), reference medium M-8 with the addition of Pluronic F68 at 12.5 CMC (M-8 + F68), NPK 8-6-6 medium (NPK) and NPK 8-6-6 medium with the addition of Pluronic F68 at 12.5 CMC (NPK + F68). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

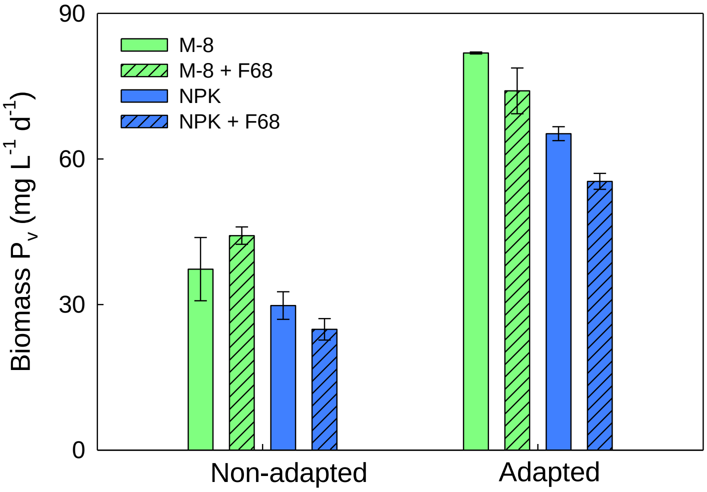


Figure 8.12. Volumetric biomass productivity of *Chlorella sorokiniana* (Biomass P_v), expressed as miligrams of biomass produced per liter of culture broth and per day, calculated at the end of the non-adapted and the adapted phase. Culture media: reference medium M-8 (M-8), reference medium M-8 with the addition of Pluronic F68 at 12.5 CMC (M-8 + F68), NPK 8-6-6 medium (NPK) and NPK 8-6-6 medium with the addition of Pluronic F68 at 12.5 CMC (NPK + F68). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

Volumetric biomass productivities were greatly improved during the repeated-batch cultivation. Productivity values were, in average, 2.1-fold higher at the end of the adaptation phase for all the conditions assayed (**Fig. 8.12**). The minor differences observed in the biomass density between cultures in the reference media and in the NPK-based media during the entire cultivation led to variations in the productivities, especially during the adapted phase. In this sense, the usage of the commercial fertilizer resulted in a 20 and 25% drop in the final volumetric productivity (without and with the surfactant, respectively) when compared to the standard media. With regards to the presence of the surfactant Pluronic F68, it led to a 15% decrease when used in combination with the NPK-based media.

According to the results, the presence of the surfactant Pluronic F68 had a minor effect (10-15% decrease) in the final productivity of *C. sorokiniana* independently of the culture media used. However, the use of the fertilizer can compromise the final productivity up to 25%. Nevertheless, the reduction in volumetric productivity caused by the commercial fertilizer is accompanied of a considerably easiness of operation and a major reduction in culture media price, which are noteworthy advantages at large scale. Estimated reference media price (calculation based on commercially available chemical reagents) is 0.231 € L^{-1} while the estimated price for the fertilizer-based media is only 0.00141 € L^{-1}. Culture medium is considered an important cost factor for microalgae production (Acién et al., 2012; Norsker et al., 2011). Therefore, the fertilizer usage can be still considered as a promising tool to achieve the sustainability of microalgae cultivation in a LF-PBR.

The posterior applications of the biomass produced will depend on the quality thereof. Previous experiments revealed that cultivation of *C. sorokiniana* in the presence of Pluronic F68 had no effect on the biochemical composition of the microalga when cultivated in liquid cultures (Chapter 7). However, the combination of the surfactant and the fertilizer may result in possible alteration of the biochemical composition, hence, the quality of the biomass produced in the presence of both the surfactant and the NPK needed to be assessed. In this sense, the photosynthetic pigments profile was evaluated during the repeated-batch cultivation. As can be seen in **Figure 8.13**, the intracellular content of chlorophyll and total carotenoids did not differ significantly among cultures in the different culture media. Both total chlorophyll and carotenoids were slightly lower in the cultures with the fertilizer but no effect due to the presence of Pluronic F68 was observed.

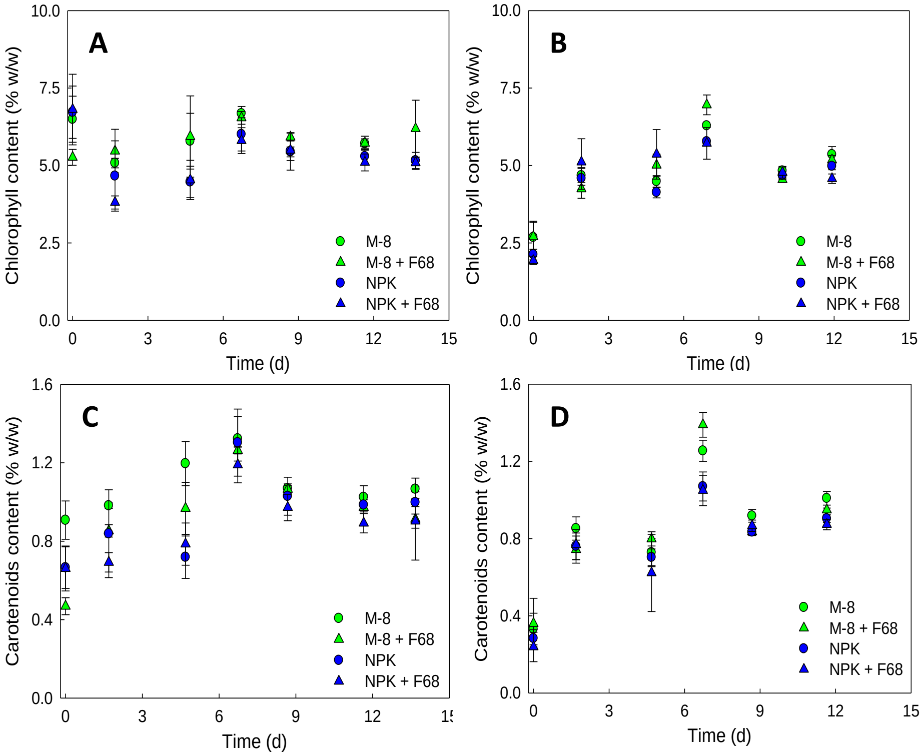


Figure 8.13. Time-course evolution of (A, B) intracellular chlorophyll (% w/w) and (C, D) carotenoids content (% w/w) of *Chlorella sorokiniana* during the non-adapted phase (A, C) and the adapted phase (B, D). Culture media: reference medium M-8 (M-8), reference medium M-8 with the addition of Pluronic F68 at 12.5 CMC (M-8 + F68), NPK 8-6-6 medium (NPK) and NPK 8-6-6 medium with the addition of Pluronic F68 at 12.5 CMC (NPK + F68). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

A deeper analysis of the methanolic extracts containing the photosynthetic pigments of *C. sorokiniana* was carried out by HPLC as described in the Materials and Methods (**Section 2.6.3**). Chlorophyll a and b, as well as lutein and beta-carotene are the main pigments present in *C. sorokiniana* (Cordero et al., 2011). Besides, lutein is also considered as an indicator of abiotic stress in microalgae (Forján et al. 2015). Therefore, the relative abundance of the main pigments was analyzed during the cultivation period.

No differences were found in the pigments profile between the different conditions assessed which also indicates absence of abiotic stress during the cultivation with the NPK supplemented with the surfactant. As example, the relative abundance of pigments at the end of the adaptation phase is shown in the figure below (**Fig. 8.14**). As can be seen, chlorophyll is the major pigment (≈ 95%

relative abundance) followed by lutein (≈ 4%). The same profile was found for all the conditions assessed which confirms no external abiotic stress was experienced by *C. sorokiniana.*

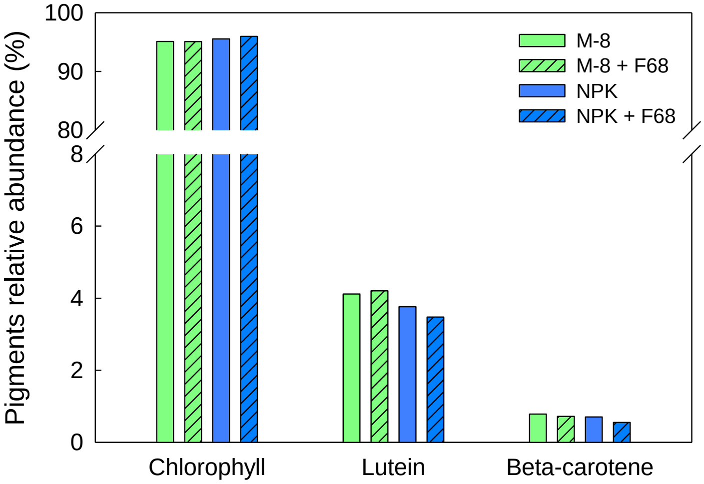


Figure 8.14. Relative abundance of main photosynthetic pigments in *Chlorella sorokiniana* (% w/w) at the end of the adapted phase. Culture media: reference medium M-8 (M-8), reference medium M-8 with the addition of Pluronic F68 at 12.5 CMC (M-8 + F68), NPK 8-6-6 medium (NPK) and NPK 8-6-6 medium with the addition of Pluronic F68 at 12.5 CMC (NPK + F68). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

The presence of the surfactant resulted in similar trends for the lutein content both in the reference cultures and the cultures with the NPK fertilizer. Although the lutein content slightly decreased in the adapted phase (data not shown), the final lutein volumetric productivity was, to some extent, higher than that of the cultures in the non-adapted phase (**Fig. 8.15**) due to the higher biomass volumetric productivity previously commented (**Fig. 8.12**). No differences were found between the reference culture and the NPK-based media, regardless the presence of the surfactant Pluronic F68.

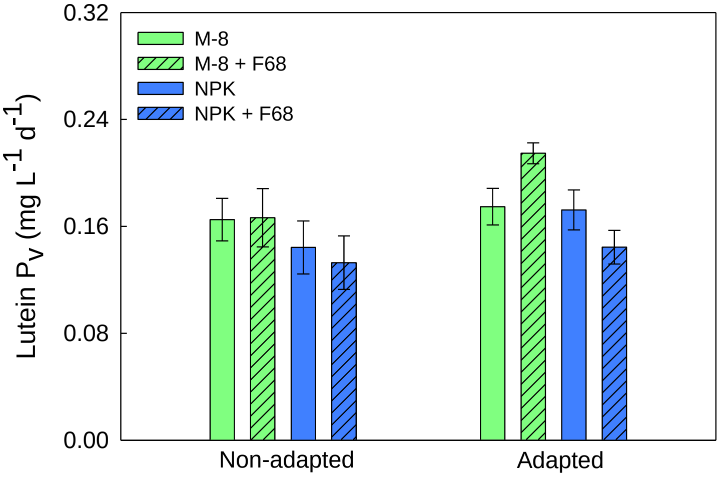


Figure 8.15. Lutein volumetric productivity of *Chlorella sorokiniana* (Lutein P_v), expressed as the miligrams of lutein produced per liter of culture broth and per day, calculated at the end of the non-adapted and the adapted phase. Culture media: reference medium M-8 (M-8), reference medium M-8 with the addition of Pluronic F68 at 12.5 CMC (M-8 + F68), NPK 8-6-6 medium (NPK) and NPK 8-6-6 medium with the addition of Pluronic F68 at 12.5 CMC (NPK + F68). The average values of duplicate cultures are shown, together with their corresponding standard deviations

In general terms, results show that biomass productivity slightly decreased using fertilizers but no influence from the surfactant presence was observed. Also no effect on the biochemical composition, evaluated as the intracellular content of photosynthetic pigments, was observed. Regarding the lutein production, the productivity values were similar under all the conditions assayed. Considering the major benefits related to media preparation and media cost, and the similar productivity values (especially lutein), it can be concluded that the use of NPK fertilizers, even in combination with the surfactant Pluronic F68, should be considered as a promising tool for the sustainable production of microalgae in foam.

8.4 Conclusions

In this Chapter, *Chlorella sorokiniana* growth was tested in modified culture media using 10 different nitrogen fertilizers, including NPK containing nitrate, ammonium, urea and mixtures of them. A correlation was found between the ureic nitrogen content of the nitrogen fertilizer and *C. sorokiniana* growth and the best performance was observed in cultures with ureic nitrogen as the sole nitrogen source.

Next, *C. sorokiniana* growth was assessed in culture media based on two NPK fertilizers containing urea as nitrogen source, but with varying NPK ratios. Although both NPKs allowed *C. sorokiniana* growth at all the concentrations tested (1-20 mL NPK L^{-1}), highest NPK biomass yields were obtained at the lowest NPK concentrations evaluated. On the other hand, low NPK concentrations (< 3mL NPK per liter of culture) led to steep pH drops due to low phosphoric acid content, which showed to be responsible for buffering the pH of the culture broth. According to the observed results, intermediate NPK concentrations should therefore be used.

Moreover, as a first approach to microalgae cultivation in surfactant-stabilized foams in fertilizers-based culture media, the feasibility of using Pluronic F68 in NPK-based culture media for *C. sorokiniana* production was proved in liquid cultures. Results show that biomass productivity slightly decreased using fertilizers but no major influence from the surfactant presence was found. Also no major effect on the biochemical composition of the microalga, evaluated as the intracellular content of photosynthetic pigments, was observed. In this sense, and regarding its potential for lutein production, the productivity values were similar under all the conditions assayed. Considering the major benefits related to media preparation and media cost, and the fact that the obtained productivity was only slightly lower, it can be concluded that the use of NPK fertilizers in combination with the surfactant Pluronic F68 can be a promising tool for the sustainable production of microalgae in foam.

Chapter 9

Assessment of surfactant effect on fatty acids extraction and cell integrity

Sections of this Chapter will be submitted as:

Vázquez M, Montero Z, Gommers E, Vílchez C, Cuaresma M. Biomass quality of microalgae cultivated in a liquid foam-bed photobioreactor prototype

9.1 Abstract

The cultivation of *Chlorella sorokiniana* in Pluronic F68-stabilized foam has been proved to be a promising cultivation concept. Besides, this surfactant has shown not to produce major changes in the biochemical composition of the biomass cultivated in the liquid-foam photobioreactor (LF-PBR). However, the interaction of surfactants with cells can result in the extraction of particular compounds. When added to liquid cultures at low concentrations, Pluronic F68 has shown to interact with several kinds of cells, mostly resulting in growth-promoting and shear-protective effects. On the other hand, other surfactants have shown to promote the extraction of lipids. Considering the effects of Pluronic F68 on the extraction of valuable algal compounds have not been studied yet, it was proposed to be addressed. Therefore, the release of fatty acids to the media broth and the cell integrity of *C. sorokiniana* were analyzed during cultivation in Pluronic F68-stabilized foams under different cultivation scenarios. Besides, the lytic effect and the capability of this surfactant to extract fatty acids were also investigated by incubation of freeze-dried *Chlorella* biomass in aqueous solutions of the surfactant at much higher concentrations than that used for cultivation in the LF-PBR.

9.2 Introduction

Cultivation in foam has been established as a promising cultivation concept with potentiality to achieve a more sustainable microalgae cultivation (Janoska et al., 2018a, 2018b; this Thesis). However, cultivation in foam implies the use of surfactants to stabilize the foam formed and, therefore, the effect of the selected surfactant over the biochemical composition of the algal biomass is required to be addressed. Previous results presented in this Thesis (Chapter 7) showed that the use of Pluronic F68 does not result in major changes in the cell composition of *Chlorella sorokiniana* during different scenarios of cultivation in foam. Nevertheless, its effect on the extraction of valuable compounds, such as fatty acids, has not been evaluated.

Interaction between surfactants and the microalgal cells can lead to alteration of the normal functioning of the cellular membranes and even to the cell membrane lysis (Ulloa et al., 2012). As example, the hydrophobic ends of certain surfactants can bind to, or insert into, the hydrophobic algal cell membranes (Lai et al., 2017). Depending on the type of interaction it may alter the biomass growth but it could also contribute to the extraction of valuable metabolites from the cells, as lipids and/or antioxidants (Glembin et al., 2014; Park et al., 2014; Ulloa et al., 2012).

Up to date, studies related to surfactants and microalgae are mainly focused on the evaluation of their toxicity when added to the culture medium but their effect as cell lytic agent has not been thoroughly studied (Ulloa et al., 2012). In particular, microalgae-derived lipids are of high interest for industry but their extraction involves high costs and high energy expenses, which are undesirable at large scale. In this sense, few works have been carried out in the last years with the aim of evaluating the extractability of lipids by mean of using surfactants (Coward et al., 2014; Glembin et al., 2014; Lai et al., 2015; Ulloa et al., 2012). As a general statement, surfactant type, critical micelle concentration as well as growth stage of biomass influence the extraction performance of each surfactant and, therefore, it needs to be particularly addressed. Non-ionic surfactants such as Triton X-100 selectively releases intracellular pigments while it had nearly no effect on intracellular lipids (Wang et al., 2015a). On the other hand, some cationic surfactants, such as CTAB, form CTAB-phospholipid micelles by its interaction with the hydrophobic components of the cytoplasmic membranes, which at the last extent results in the solubilization of the microalgal cell membrane components (Gilbert and Moore, 2005; Huang and Kim, 2013). In this sense, *Chlorella* biomass harvested by CTAB-aided foam flotation has shown to increase the lipid recovery compared to centrifugation (Coward et al., 2014). This was partially explained by the adsorption of the surfactant onto the algal cells and its subsequent presence in the lipid extraction process, but also related to the cell lysis and in situ solubilization of the algal cell membrane, which favored the extraction of lipids. Besides, the lytic effect and the extractability capacity of a surfactant could depend on the concentration employed. For instance, the cationic surfactant myristyltrimethylammonium bromide (MTAB) can result in the release and solubilization of extracellular polymeric substances of *Synechocystis* or can cause cell lysis depending on the concentration employed (Zhou et al, 2017).

Pluronics are bioactive surfactants that can interact with biological membranes (Doğan et al., 2012). However, specific Pluronic subtypes exhibit distinct properties. In particular, Pluronic F68 has been shown to interact with several types of cells, including yeast, plant and animal cells, leading to a variety of effects among which the most common ones are protection from mechanical and chemical stress and growth promotion (Lowe et al., 1994; Ntwampe et al., 2010). As example of application in microalgal cultures, a recent study showed that Pluronic F68 reinforces the cell integrity of *Chlamydomonas reinhardtii* when exposed to a toxic substrate that damages the cell membrane (Zhao et al., 2016). Pluronic F68 molecules contain polyol groups, and it was hypothesized that the aforementioned protective effect is related to the formation of a polyol layer on the cell surface (Murhammer and Gooche, 1988). However, it has been shown that Pluronic F68 can insert into the cell membrane and enter the cells (Gigout et al., 2008) and it has been suggested that Pluronic F68 preferentially interacts with damaged cell membranes (Hädicke and Blume, 2013). Therefore, the effects of Pluronic F68 on the cell integrity and the extraction of valuable compounds may be strengthened if the cell robustness has been previously diminished, for instance due to the exposure to stress conditions or caused by treatments like freeze-drying.

The interactions of Pluronics with cell membranes have been further investigated beyond the abovementioned effects reported on some cell cultures. In this sense, several studies have been performed in order to study the interactions of several

Pluronics with artificial phospholipid monolayers, which serve as a model of cell membranes (Chang et al., 2005; Hädicke and Blume, 2013). It was observed that the higher the hydrophobicity of the Pluronic, the larger the surface activity and the ability to penetrate the membranes (Chang et al., 2005). These studies suggested that the interactions of these surfactants with phospholipid monolayers may be based on van der Waals forces and hydrophobic interactions. Conversely to the protective effects abovementioned, there exist also reports about the opposite effect, namely bilayer permeabilization and disruption of bilayers above the critical micelle concentration (Hädicke and Blume, 2013). Experiments performed in lipid monolayers suggested that the hydrophobic interactions of the polypropylene oxide (PPO) blocks of the Pluronics provide the driving force for the interaction, but the polyethylene oxide (PEO) blocks also interact with the lipid layer, possibly by intercalating in between the lipid head group regions. Furthermore, it has been suggested that Pluronic F68 causes the formation of transmembrane pores in artificial lipid bilayers, possibly by affecting both lipid-lipid and lipid-protein interactions (King et al., 1991). This was the explanation considered by King and co-workers (1991) for the increased uptake of a fluorescent dye found by yeast cells due to the addition of Pluronic F68 to the cultures. Consequently, products release during cultivation in foam might be expected as a result of the surfactant interaction with the microalgal cell membranes.

In this study, the effect of Pluronic F68 over *C. sorokiniana´s* cell integrity was examined by scanning electron microscopy (SEM), in order to unveil the effect of Pluronic over the cellular morphology and/or integrity. Moreover, during the different cultivation experiments in foam, the fatty acid content was analyzed in the biomass pellet as well as in the total culture broth in order to determine the possible release of some fatty acids to the broth during cultivation in foam. These effects were studied for different cultivation scenarios in foam, including nitrogen starvation and saturating light intensities. Finally, the capability of the surfactant to extract fatty acids and its lytic effects were also investigated by incubation of freeze-dried *Chlorella* biomass in aqueous solutions of the surfactant at much higher concentrations (up to 10-fold the concentration used during the cultivation).

9.3 Results and discussion

9.3.1 Assessment of Pluronic F68 effect on fatty acids extraction during cultivation in foam

Cultivation in surfactant stabilized-foam results in a continuous exposure of microalgal cells to the selected surfactant. With the aim of revealing whether extraction of lipids was occurring during the cultivation in foam, the fatty acid content was analyzed in the biomass pellet as well as in the total broth (containing the supernatant) (**Fig. 9.1**). Total broth and pellet samples were freeze-dried previous fatty acids extraction in order to remove all the water contained in the different fractions. Differences in the fatty acid content between both fractions could be used, if occurring, as estimation of the amount of fatty acids released to the media.

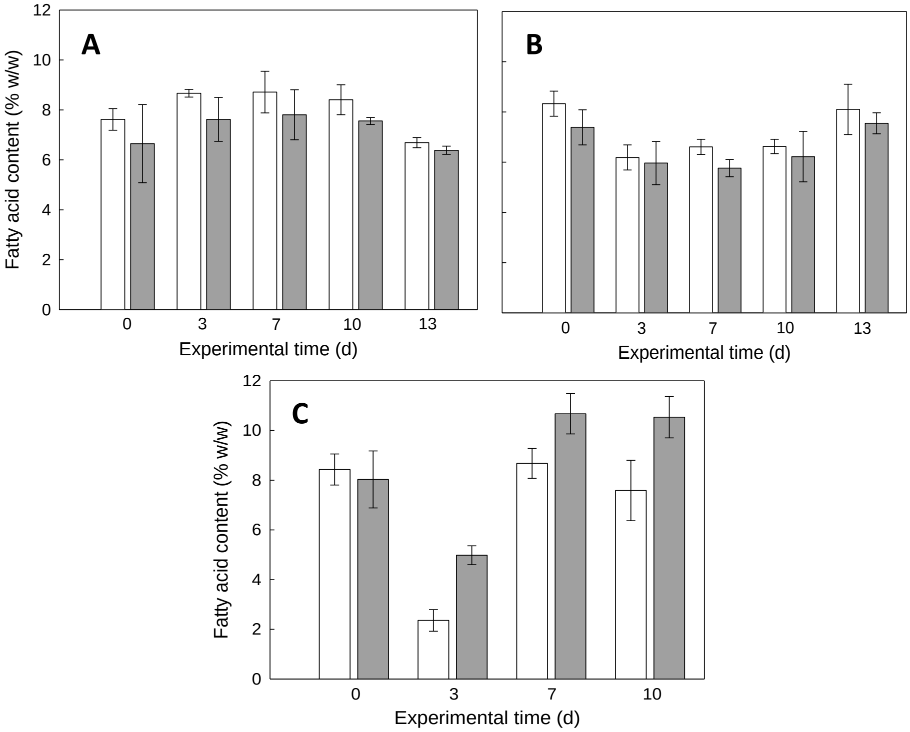


Figure 9.1. Evolution of fatty acid content of *Chlorella sorokiniana* (% w/w) contained in the biomass pellet (white bars) and in the total broth (grey bars) during the different cultivation scenarios in foam: (A) 3-fold concentrated M-8 culture medium, (B) 3-fold concentrated M-8 culture medium under saturating light intensity and (C) nitrogen-starved cultures. The surfactant Pluronic F68 was added at the concentration of 12.5 CMC (i.e. 4.175 g L^{-1}). The average values of duplicate cultures are shown, together with their corresponding standard deviations.

Figure 9.1 shows the results corresponding to three different scenarios of *C. sorokiniana* cultivated in foam: 3-fold concentrated medium, 3-fold concentrated medium under saturating light intensity and nitrogen-starvation. Results related to microalgal growth and the biochemical composition of the biomass cultivated in these scenarios were presented in Chapter 7. It can be inferred from the figure that there were no significant differences between the fatty acid content in the pellet and in the total broth during cultivation in 3-fold concentrated media (**Fig. 9.1A**) as well as during the cultivation with saturating light (**Fig. 9.1B**). However, cultivation under nitrogen starvation revealed a slightly higher fatty acid content in the total broth (**Fig. 9.1C**) which might be related to some fatty acids released to the media. Since cultivation in Pluronic F68-stabilized foams under the other conditions assessed (**Fig. 9.1A, B**) did not result in the release of fatty acids, the fatty acids release observed under nutrient starvation might be related to the physiological consequences of such growth inhibiting condition. Nutrient starvation compromised cell viability (Chapter 7), possibly resulting in a higher extent of cell death and the consequent release of fatty acids to the broth.

In order to perform a deeper analysis of the differences found between the pellet and the total broth during the cultivation in foam under nitrogen starvation, the fatty acids profiles were also analyzed. **Figure 9.2** shows the specific fatty acid content, as percentage of biomass dry weight (**Fig. 9.2A**), and the relative abundance of fatty acids in the pellet and in the total broth at day 7 (**Fig. 9.2B**), when fatty acid content was maximal.

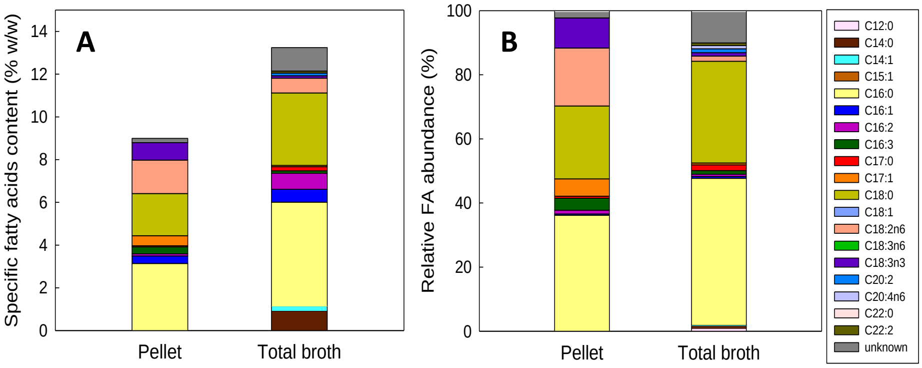


Figure 9.2. (A) Specific fatty acids content (% w/w) and (B) relative abundance of fatty acids, expressed as percentage of total fatty acids, contained in the pellet and in the total broth during the cultivation of *Chlorella sorokiniana* in foam under nitrogen starvation at day 7.

Main differences between both fatty acid profiles are related to the saturated fatty acid content. C14:0, C16:0 and C18:0 content in the total broth are higher than in the pellet (**Fig. 9.2B**), which resulted in an increase in their relative abundance of 26% and 39% for C16:0 and C18:0. The specific content of C16:2 also showed a 7.5-fold higher content in the total broth. It seems to indicate that such fatty acids were the major fatty acids to be released to the supernatant. On the other hand, the content of C17:1 and C18:3n3 was lower in the total broth samples. This might be explained by the retention of these fatty acids by the surfactant during the fatty acid extraction method. During the lipids extraction, the surfactant was present in the total broth samples, while it was mostly removed in the pellet samples by the centrifugation step. As a consequence, Pluronic F68 might have retained some of the fatty acids from the total broth samples (i.e. C17:1 and C18:3n3) in the interface formed during the lipids determination performance, possibly explaining the lower content of these fatty acids in the total broth.

Under nutrient starvation conditions, microalgae in general divert the synthesis of new membranes to the conversion of fatty acids into triglycerides. It serves as mechanism for channeling the large amounts of ATP and NADPH which under standard conditions should have been used for cell growth. Consequently, triglycerides can temporarily accumulate and can be utilized further as an energy-rich carbon source when conditions for growth improve (Thompson, 1996). However, prolonged cultivation under nutrient starvation may lead to cell viability loss and, therefore, cell death with the corresponding liberation of intracellular metabolites to the broth.

Several studies suggest that *Chlorella*'s cell integrity is not affected by nitrogen starvation in liquid cultures. It is shown by an increase in cell size and cell wall thickness, as well as by an unaltered resistance to mechanical disruption (Martin et al., 2014; Yap et al., 2016). Our results showed that cultivation in foam during nitrogen starvation strongly compromised the photosynthetic capacity of *C. sorokiniana* (Chapter 7) and it resulted in an important cell viability loss. Cell death under such conditions occurred and, as a consequence, liberation of intracellular metabolites could possibly take place. Next to some fatty acids (i.e. C14:0, C16:0, C16:2 C18:0), the released intracellular metabolites may include surfactant-like molecules (e.g. proteins), which during the lipids extraction method might have contributed to the retention of other specific fatty acids, thus explaining their decrease in the total broth samples (i.e. C17:1 and C18:3n3).

In order to unveil the effects of cultivation in foam on cell morphology and/or integrity, samples were taken during the experiments carried out in foam and were analyzed by scanning electron microscopy (SEM). The micrographs (**Fig. 9.3**) confirmed that the integrity of *C. sorokiniana* cells was maintained under all the experimental conditions assayed, except for the combination of nitrogen starvation and illumination with saturating light (**Fig. 9.3I, J**). Also cell size (between 2 – 3 µm) and cell morphology remained unchanged.

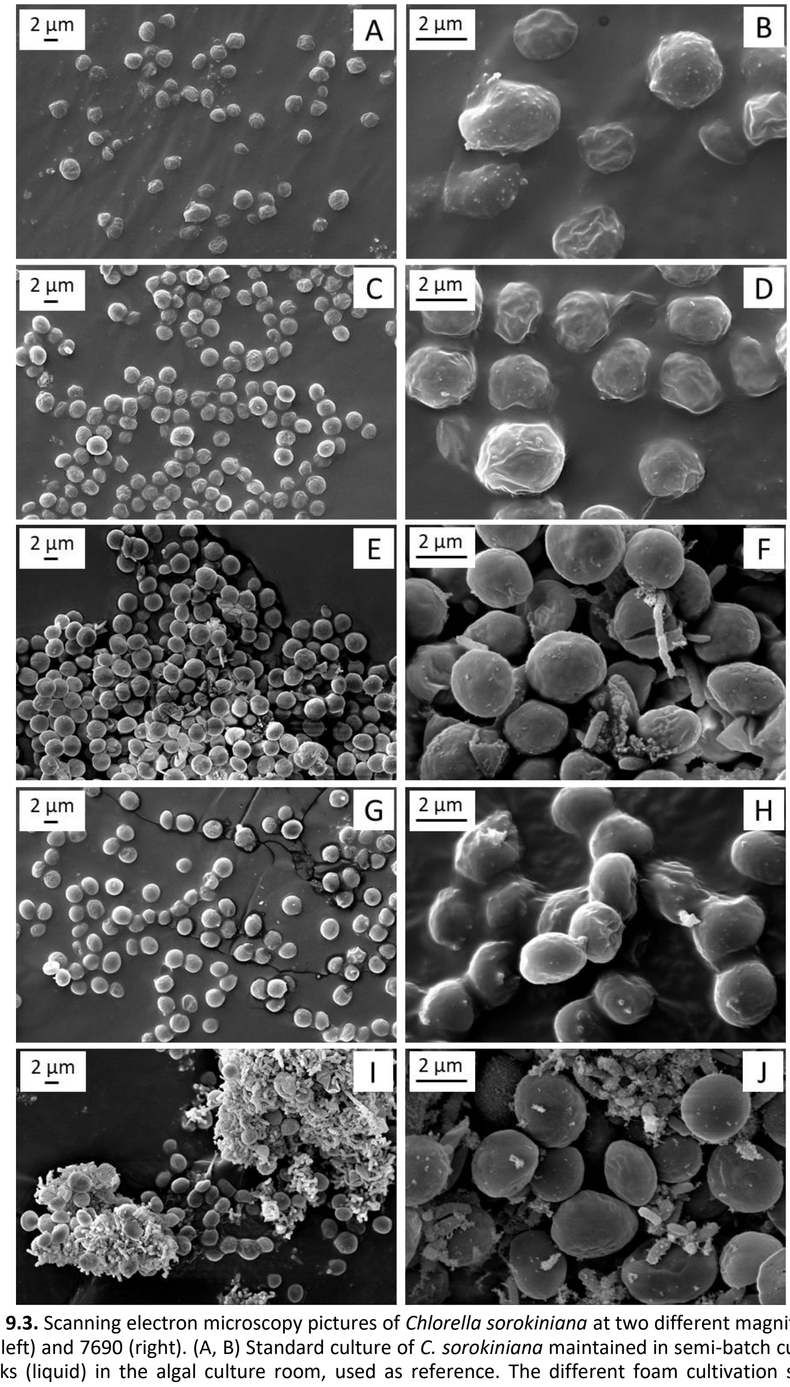


Figure 9.3. Scanning electron microscopy pictures of *Chlorella sorokiniana* at two different magnifications: 1980 (left) and 7690 (right). (A, B) Standard culture of *C. sorokiniana* maintained in semi-batch cultivation in flasks (liquid) in the algal culture room, used as reference. The different foam cultivation scenarios were: (C, D) 3-fold concentrated M-8 culture medium, (E, F) N- starvation, (G, H) 3-fold concentrated M-8 culture medium under saturating light intensity, and (I, J) N-starvation combined with saturating light. The samples were obtained at the end of the cultivation period, which was 13 days (C, D, G, H), 10 days (E, F) and 48 hours (I, J). Scale bars are present in each picture.

Particularly, under nitrogen starvation most of the cells showed small round protuberances on their surface (**Fig. 9.3E, F**). Although scanning electron microscopy does not allow to distinguish intracellular structures, the laser beam can penetrate up to 20 nanometers, depending on the surface characteristics. It may explain that starch globules which are massively accumulated under nitrogen starvation in *Chlorella* cells, could be seen as small round protuberances with a lighter electron density. It would be in agreement with the high carbohydrate content observed in *C. sorokiniana* cultivated in foam under nitrogen starvation (Chapter 7) but also with previous studies where a high accumulation of starch globules was observed in *Chlorella* sp. cells under transmission electron microscopy as white deposits (Cheng et al., 2017; Tanadul et al., 2014; Zachleder and Brányiková, 2014).

Apart from the unique morphology observed under nitrogen starvation, some cellular debris and a low bacterial content was observed in the samples (**Fig. 9.3E, F**), which is consistent with the hypothesis of a certain degree of cellular death which can result in the liberation of fatty acids to the culture broth. Besides, when nitrogen depletion was combined with saturating light intensity the culture collapsed at 48 hours (Chapter 7). Under such conditions, the SEM picture revealed an important content of cellular debris as well as proliferation of bacteria (**Fig. 9.3I, J**) as a consequence of the massive cellular death.

9.3.2 Assessment of extractant and lytic capabilities of Pluronic F68 on microalgal cells

The biocompatibility of Pluronic F68 with *C. sorokiniana* was proved in the previous section of this Chapter, since it did not affect the cell integrity, nor did it produce the release of fatty acids during cultivation in foam in nutrient-replete medium. However, few works have demonstrated the capability of certain surfactants to extract lipids (Glembin et al., 2014; Lai et al., 2015; Park et al., 2014). In this sense, it was decided to evaluate the capability of Pluronic F68 to extract fatty acids when used at much higher concentrations than the one used for cultivation (up to 125 CMC).

The effect of Pluronic F68 on the extraction of fatty acids from *C. sorokiniana* biomass was assessed at concentrations ranging from 0 to 10-fold the concentration used for cultivation in foam (0 CMC, 12.5 CMC, 31.25 CMC, 62.5 CMC, 125 CMC, with CMC_{F68} = 0.334 g L^{-1}). In order to provide a better understanding, the experimental set-up is schematically described in **Figure 9.4**. Solutions containing the different concentrations of Pluronic F68 were prepared in distilled water, and 5 mL of the corresponding surfactant solutions were added to 5 mg of dry biomass in order to keep the concentration of the surfactant but also the biomass quantity. The biomass used in the experiments was taken from a liquid culture of *C. sorokiniana* grown under nitrogen deprivation for 3 days in order to promote a slightly higher fatty acid content in the cells, which could increase the reliability of the obtained data. Nevertheless, it must be noted that cultivation in foam under nitrogen starvation during 10 days did not to increase the fatty acid content of the biomass (approx. 10%) (Chapter 7, **Fig. 7.6C** and **Fig. 9.1**), however, this was unknown at the time of the experimentation. Finally, nitrogen starvation seems not to compromise cell integrity in liquid cultures (Chapter 7) and, therefore, the results might not be altered by the quality of the biomass used.

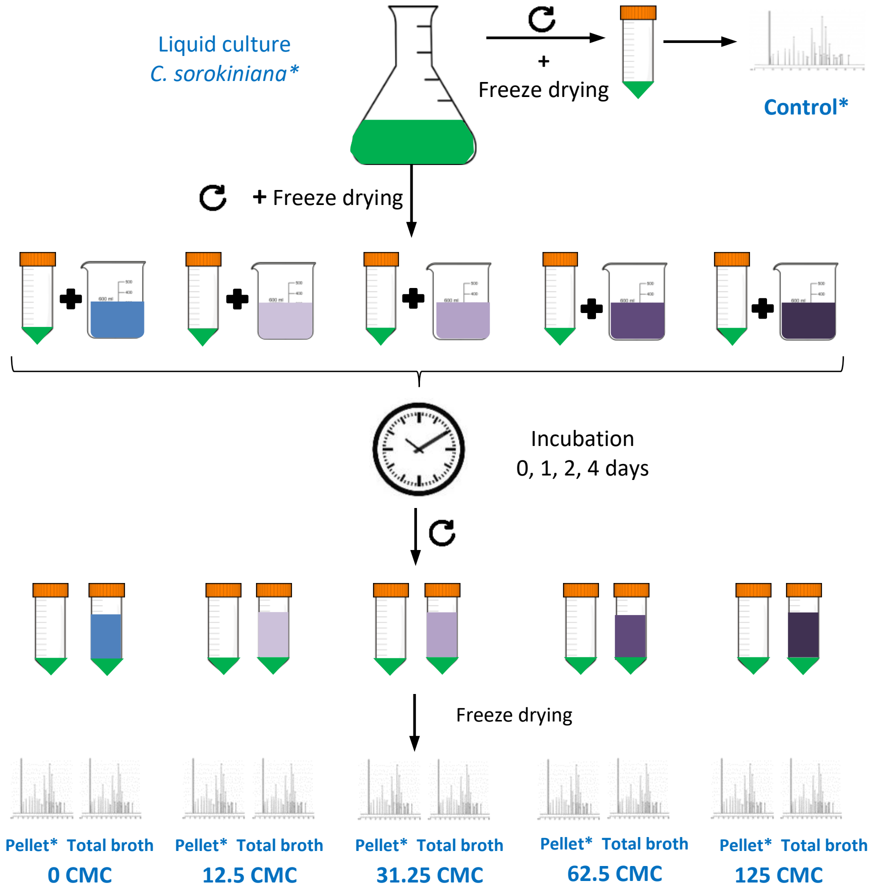


Figure 9.4. Schematic drawing of the experimental set-up followed for the evaluation of the extractability of fatty acids by Pluronic F68 employed at high concentration. (*) represents the samples which were also analyzed by scanning electronic microscopy (SEM).

The assay was similar to that performed by Lai and co-workers (2015), in which several surfactants were assessed as enhancers of fatty acids recovery from algal biomass. For the present assay, the biomass samples were centrifuged and freeze-dried before incubation with the different surfactant solutions. For the samples incubated without the surfactant (0 CMC), 5 mL of distilled water were added after freeze-drying. After re-suspension of the dry biomass in the different solutions by vortexing, the tubes were tightly closed and incubated in an orbital shaker at 100 rpm at room temperature. Samples at different incubation times were taken (0, 1, 2 and 4 days). In order to determine the remaining fatty acids in the pellet, the samples were centrifuged (at 3000 g for 5 minutes) and the pellet was freeze-dried prior to the fatty acids extraction and methylation. For the analysis of the fatty acid content in the total broth, not centrifuged sample replicates (containing the biomass as well as the supernatant) were also freeze-dried prior the fatty acids extraction and methylation. The fatty acids were extracted, trans-esterified and analyzed as explained in Materials and Methods (**Sections 2.6.5** and **2.6.6**). In all cases, duplicate samples were analyzed.

The analysis of the fatty acid content in the biomass pellet and in the total broth, after the different incubation times and for the different Pluronic F68 concentrations tested, is shown in **Figure 9.5**. In order to have a reference value of the fatty acids contained in the biomass used in the experimental set-up, a Control sample was analyzed. Such Control sample was directly taken from a liquid culture of *C. sorokiniana* incubated in the algal culture room, it was freeze dried and fatty acids were directly extracted and methylated without any previous treatment (**Fig. 9.4** and **9.5**, referred as Control). Apart from that reference, it was decided to have another reference in order to see if the experimental procedure (incubation in aqueous solution + 2° freeze drying step) resulted in any effect over the fatty acids released. For that purpose, biomass samples incubated with water were also prepared and evaluated (**Fig. 9.4** and **9.5**, referred as 0 CMC). Differences observed between the fatty acid content of the samples incubated with water (0 CMC) and the ones incubated at different surfactant concentrations may be the result of the extra effect of the surfactant over the extraction of fatty acids.

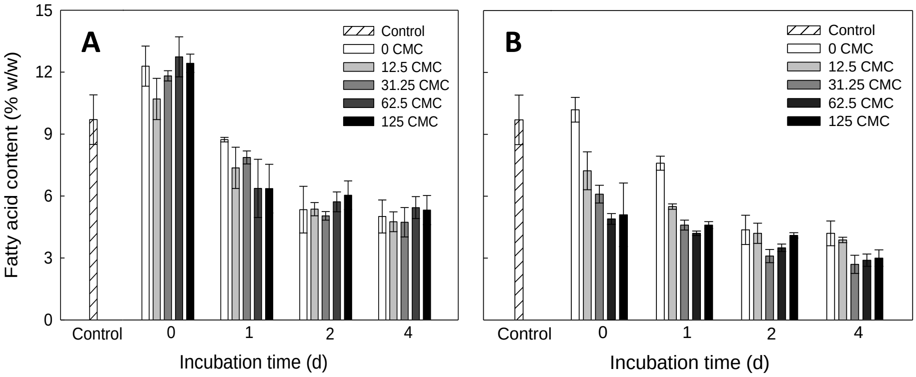


Figure 9.5. Fatty acid content of *Chlorella sorokiniana*, expressed as percentage of biomass dry weight, measured (A) in the pellet and (B) in the total broth at different incubation times (0, 1, 2 and 4 days) and for the different surfactant concentrations assayed. Error bars show the standard deviation of duplicate samples. Control represents the fatty acid content of samples obtained from the liquid culture without being incubated in any solution and freeze-dried only once.

Maximal fatty acid content in the pellet was found in the samples which followed the experimental procedure without any incubation time (**Fig. 9.5A**, 0 days) but no significant differences were found between treatments at that moment. Compared to the Control (no incubated and freeze dried only once), the fatty acid content was, in average, a 25% higher when the samples followed the experimental procedure (which implied the addition of water/Pluronic, centrifugation and a second freeze drying step). Considering there were no differences between the samples containing water and the ones containing Pluronic, it can be concluded that the increase in the amount of fatty acids measured was related to the second freeze drying step to which the samples were exposed. Freeze drying might, therefore, alter cell integrity and facilitate the extraction of the fatty acids from the biomass.

Regarding the pellet samples incubated during 1, 2 and 4 days, also no significant differences were found between the treatments at each incubation time (with and without Pluronic) (**Fig. 9.5A**; 1, 2, 4 days). However, it was observed that incubation time resulted in decreasing fatty acid contents, achieving a minimal value after 2 days of incubation. Considering freeze drying process alters cell integrity, the incubation at room temperature in an orbital shaker during at least 1 day might imply an extra mechanical stress to the biomass and metabolites as fatty acid could be released to the broth. It could explain the lower fatty acid content observed in the pellet during the incubation.

The fatty acid content analyzed in the total broth (**Fig. 9.5B**) also revealed an interesting trend. On one hand, the absence of incubation resulted in values similar to those for the Control when water was used, but samples with Pluronic already showed a lower fatty acid content. A similar value, or slightly higher, to the one obtained for the pellet might be expected without incubation. However, the lower values obtained can be explained by the methodology employed to extract and methylate the fatty acids. Samples from the total broth contained the algal biomass plus other components which were already released to the broth as well as the surfactant. After freeze drying, all these components remained in the samples, while pellet samples lost most of these components and the surfactant during the centrifugation step previous to the freeze drying (**Fig. 9.6A**). The existence of such components, surfactant and other intracellular surfactant-like molecules, resulted in the formation of an interface during the fatty acids extraction protocol (**Fig. 9.6B**), which is not present when the samples are free of surfactant. Such emulsion might be responsible for retaining part of the fatty acids of the sample in the interface and therefore avoiding them to be part of the bottom phase which is further methylated. It could explain the lower fatty acid content found in the total broth samples when the surfactant was present.

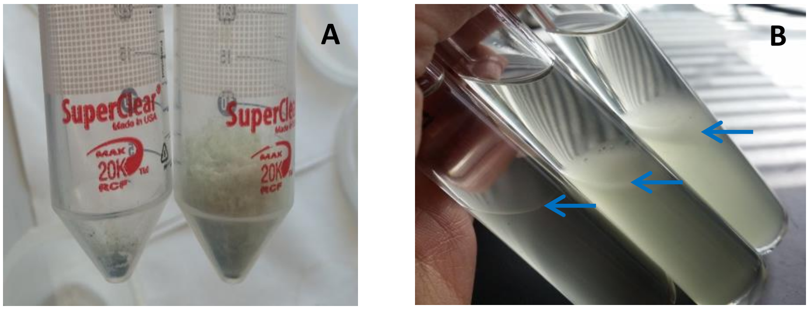


Figure 9.6. (A) Freeze dried *Chlorella sorokiniana* samples containing Pluronic F68 at 12.5 CMC (left tube: pellet sample, right tube: total broth sample). (B) Samples during the lipid extraction protocol showing interphases of different thickness (arrows) related to the surfactant presence.

However, also samples incubated with water during at least 1 day showed a lower fatty acid content compared to the control. Although Pluronic was not present in such samples, cell integrity has been proved to be totally altered with the second freeze drying step plus the incubation in the orbital shaker and, therefore, intracellular components might have been released to the broth. It is known that certain metabolites such as proteins or exopolysaccharides can have surfactant properties (Zhou et al., 1998; Žutić et al., 1981) and, consequently, they might be responsible for retaining fatty acids as previously explained. It was corroborated with the observation of a small interface formed during the fatty acids protocol, as well as it occurred with the samples with Pluronic, although in this case the emulsion was smaller (data not shown).

Apart from the analysis of the fatty acid content, samples were taken to be analyzed by SEM. Same procedure as previously described was followed for the sample preparation. In order to evaluate the effect on cell integrity and/or morphology a control culture of *C. sorokiniana* cultivated in liquid under standard conditions was used as reference (**Fig. 9.7A**). As already mentioned, it was suspected than freeze drying might alter the cell integrity and, therefore, it may explain the higher fatty acids release to the broth. It was confirmed by the SEM pictures, in which the same liquid culture after freeze drying showed a higher degree of cell breakage and cell debris (**Fig. 9.7B**). It has to be noted that both pictures were taken from biomass not exposed to Pluronic F68, so the effect on cell integrity was exclusively produced by the freeze-drying process.

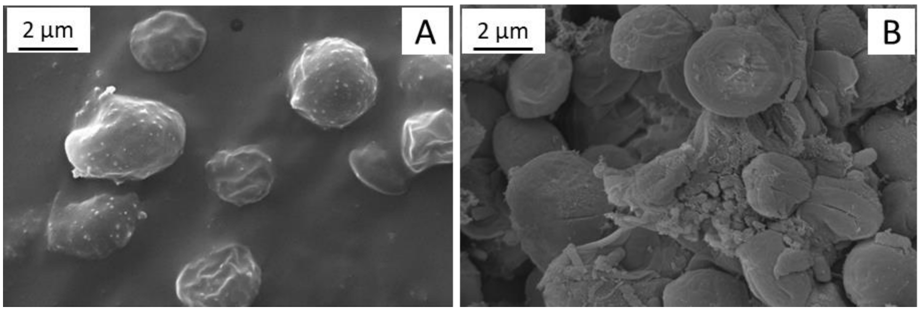


Figure 9.7. Scanning electron microscopy pictures of *Chlorella sorokiniana* at a magnification of 7690. (A) A standard culture of *C. sorokiniana* cultivated in (liquid) is shown and compared with (B) the same culture after freeze drying. Scale bars are present in each picture.

In order to unveil the possible extractant effect of Pluronic F68, the rest of the samples were also analyzed by SEM (**Fig. 9.8**). Under the same conditions, incubation time resulted in a major degree of cell disintegration and, therefore, a much higher content of cells debris (comparing columns 1 and 2 of **Fig. 9.8**). The mechanical stress imposed by the mechanical agitation to which the samples were exposed during the incubation, together with the weakness of the cells after the second freeze drying step, resulted critical for the cell integrity.

Regarding the effect of the surfactant, samples exposed to different concentrations of the surfactant (**Fig. 9.8B** and **9.8C** as example) showed no significant differences, although a slightly higher degree of cell lysis was observed at the major surfactant concentration used. However, no big differences were found with respect to the samples incubated in water only which confirms the absence of a major effect from Pluronic F68 on cell integrity.

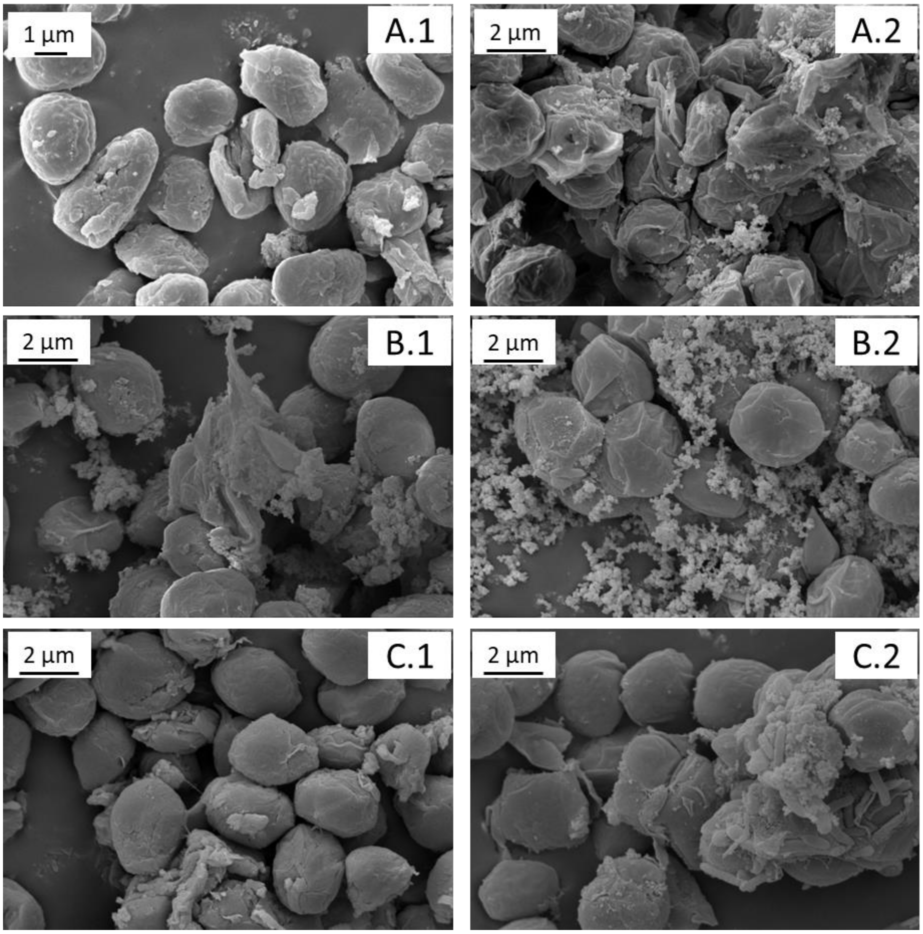


Figure 9.8. Scanning electron microscopy pictures of *Chlorella sorokiniana* at a magnification of 7690 after following the experimental extraction set-up without incubation (left column, 1) and after an incubation time of two days (right column, 2): (A) Water, (B) 12.5 CMC Pluronic F68 and (C) 125 CMC Pluronic F68. Scale bars are present in each picture.

9.4 Conclusions

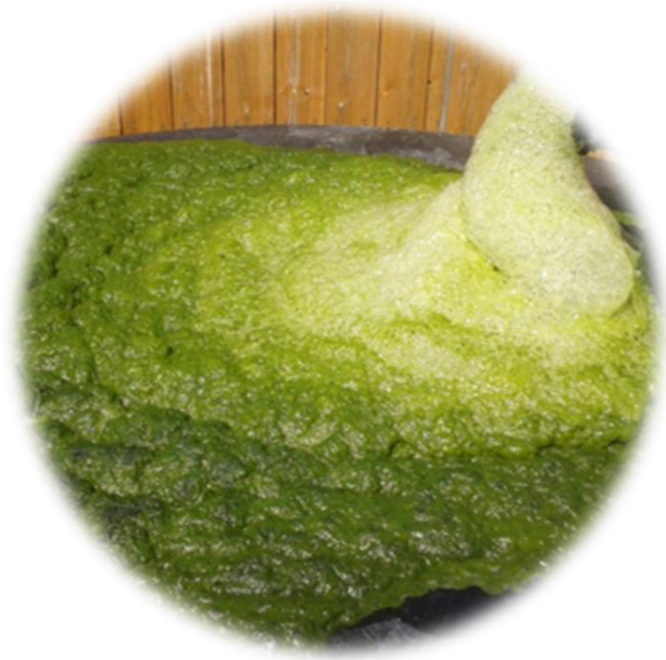
The integrity of *Chlorella sorokiniana* cells during its cultivation in Pluronic F68-stabilized foams was assessed based on the release of fatty acids to the culture broth and the analysis of the cell morphology by SEM. Up to 13 days of cultivation, the surfactant did not facilitate the extraction of fatty acids in foam. Scanning electronic microscopy (SEM) observations confirmed that the integrity of *C. sorokiniana* cells remained unaltered during cultivation in Pluronic F68-stabilized foams. A minor fatty acids release to the culture broth was only observed under nitrogen starvation, which was most probably related to a certain degree of cell death under such conditions, as a consequence of the compromised photosynthetic efficiency. Pluronic F68 used at a concentration of 12.5 CMC showed, therefore, a good biocompatibility with *C. sorokiniana* in foam-based cultures.

Moreover, the capability of this surfactant to extract fatty acids was also investigated by incubation of freeze-dried *Chlorella* biomass in aqueous solutions of the surfactant at concentrations up to 10-fold higher than that used for cultivation in the LF-PBR. Although certain fatty acid release was observed, it was most probably related to the effects of the freeze drying and incubation rather than to the surfactant, since no differences were observed between incubation with water or Pluronic F68. Furthermore, the absence of a major effect of Pluronic F68 on *C. sorokiniana* cell integrity was confirmed by SEM observations.

Altogether, it could be concluded that Pluronic F68 is biocompatible with *C. sorokiniana* and it does not affect cell integrity even at high concentrations. However, it has to be addressed that Pluronic F68 can interfere with the fatty acids released to the broth, which can be considered a drawback for the analysis of fatty acids in the total broth but also a tool to recover lipids from the supernatant. The later might require further investigation.

Chapter 10

General discussion and Future prospects



10.1 Microalgae cultivation in liquid foams

The rapidly increasing global population combined with the excessive consumption of the limited fossil fuels are some of the reasons behind the need to transition to a circular bioeconomy, an economic concept where renewable materials are used as the feedstock for fuel and chemical production while all the byproducts are completely biodegradable (Gifuni et al., 2019). In this sense, microalgae have gained attention as a promising renewable feedstock; not only to produce biofuels but also chemicals with different market applications (Acién et al., 2014; 't Lam et al., 2018; Wijffels and Barbosa, 2010). Although microalgae have been used by humans for more than 2000 years due to their nutritional value, microalgal biotechnology only started to develop by the mid-50s (Spolaore et al., 2006). Since then, a wide range of potential applications and derived products obtained from microalgae have been explored, but the commercial applications of microalgae on an industrial scale are still scarce due the still high production costs. In order to achieve the economic feasibility of more microalgae-based production processes, algal cultivation systems still need to be further improved (Spolaore et al., 2006). So far, microalgae are commercially produced in open and closed photobioreactors, where microalgae grow in liquid suspensions in which water have to be separated from the algal biomass afterwards through a costly and energy consuming process, mostly centrifugation. In this sense, new cultivation systems with advantages to overcome the current limitations of conventional cultivation systems, including reduction in water and energy consumption and its posterior expensive separation from the algal biomass, are also worthy of investigation.

In this context, a microalgae cultivation concept based on surfactant-stabilized liquid foams was recently proposed (Janoska et al., 2017). Compared to liquid-based algal cultivation, cultivation of microalgae in foam presents several advantages, some of them shown in this Thesis work. Among the advantages, cultivation at high biomass densities, which results in high productivities, and a reduction of the posterior harvesting costs can be highlighted. Despite the newness of the concept, the few studies reported on the subject, including the results obtained in this Thesis, so far suggest that cultivation of microalgae in foam could become a suitable alternative for cost-effective microalgae-based production processes (Janoska et al., 2017, 2018b).

The results obtained in this Thesis provide insight into the novel concept of cultivating microalgae in surfactant-stabilized liquid foams. Besides, some guidelines for future research on foam-based microalgae cultivation are hereby provided. The particular aspects addressed in this Thesis are discussed in greater detail below.

10.2 Stable and viable microalgal-enriched foam as cultivation matrix

One of the key aspects to achieve stable microalgae cultivation in foam is finding a microalga-surfactant combination that is suitable for this purpose. Before the idea of cultivating microalgae in liquid foams arose, the study of microalgal-enriched surfactant-stabilized foams had been limited to harvesting purposes, where the suitability of a particular alga-surfactant combination mainly depended on the extent at which the foam is enriched in algal biomass. This aspect was included in this Thesis as one of the selection criteria used to assess the suitability of microalgae and surfactants to be employed in a liquid foam-bed photobioreactor (LF-PBR). However, in this Thesis it has been established that a combination of factors regarding not only the characteristics of the algal strain and those of the surfactant, but also aspects related to the microalga-surfactant combination employed, have to be taken into consideration for the aforementioned assessment. In particular, the lack of toxicity of the surfactant to the microalga cultivated and its resistance to biodegradation are important aspects to be taken into account for the selection of microalga-surfactant combinations with potential to be used in a LF-PBR.

Before this Thesis, only one microalga-surfactant combination had been experimentally demonstrated to be suitable for microalgae cultivation in a LF-PBR. This was achieved by Janoska and co-workers (2017) for *Chlorella sorokiniana* in foams stabilized by bovine serum albumin (BSA), but due to denaturation and biodegradation of the protein, the cultivation process was limited to periods of a few hours. In this Thesis, the guidelines to independently select algal strains and surfactants with potential to be used in a LF-PBR are provided. The guidelines were proposed upon assessing a certain combination of characteristics of the algal species or the surfactant. By doing so, some promising algal strains and surfactants were selected and allowed to find a suitable microalgal-surfactant combination for its application in a LF-PBR in a stable and successful way. The suitability of this combination was experimentally demonstrated in this Thesis (Chapter 7) and in a recent independent study (Janoska et al., 2018a). Therefore, this suitable microalga-surfactant combination could be used to perform further research on the topic. For instance, it could be useful to test new LF-PBR designs or to scale up the cultivation of microalgae in surfactant-stabilized foam in future attempts.

Regarding the search of further suitable microalgae-surfactant combinations, it must be noted that the addition of a surfactant is needed to evaluate most of the criteria related to the potential of a microalgal strain and vice versa. The criteria hereby considered for the selection of microalgae with potential to be cultivated in foam were: (i) the surfactant-mediated foamability, (ii) the microalgal partitioning towards the foam phase, (iii) the stability of the foam formed, and (iv) the inherent growth characteristics of the algal strain. Except for the last one, the rest of parameters required the addition of a surfactant in order to be measurable. On the other hand, it was concluded that the potentiality of a surfactant to be employed in a LF-PBR should be evaluated according to: (i) its foaming properties, (ii) the microalgal partitioning towards the foam formed, (iii) its resistance to biodegradation, and (iv) its toxicity to microalgae. Again, with the exception of the foaming properties, the presence of microalgae or the bacteria associated to algal cultures is needed to be considered to properly assess the rest of surfactant selection criteria. Although the suitability of different algal strains and surfactants were hereby investigated independently by using model surfactants and algal strains, respectively (Chapter 3 and 4), the fact that the suitability of a particular microalga-surfactant combination have to be jointly approached is noteworthy. As a hypothetical example, when assessing the suitability of a surfactant for its application in a LF-PBR, a surfactant with good foaming properties and a high resistance to biodegradation may also allow a high microalgal partitioning towards the foam phase while lacking toxicity for the model microalgal strain used. Thus, this surfactant would be deemed as a suitable candidate. But such surfactant may be toxic for other algal strains, therefore making its application unsuitable when combined with those microalgae.

Next to assessing the suitability of a microalgae and surfactants in a jointly manner, from this Thesis it can be inferred that all selection criteria should be collectively evaluated, since they are often negatively correlated. For instance, due to the negative charge of the microalgal surfaces, cationic surfactants are the best in terms of algal enrichment in the foam. However, cationic surfactants are in general more toxic to algae than their anionic and nonionic counterparts (Chapter 4; Liang et al., 2013). As a particular example, cetyltrimethylammoium bromide (CTAB) is a cationic surfactant with proved efficiency to collect microalgae by foam flotation (Coward et al., 2014; Garg et al., 2014; Laamanen et al., 2016), but its high toxicity to microalgae has been repeatedly reported (Bogatirev et al., 2015; Liang et al., 2013; Poremba et al., 1991; Chapter 4). For this reason, this cationic surfactant was considered unsuitable for its application in a LF-PBR in this Thesis; despite it showed the best performance of all tested surfactants in terms of algal enrichment in the foam formed (Chapter 4).

Another example of the fact that all selection criteria must be jointly taken into consideration is the biodegradable character of a surfactant and its toxicity. There are many reported studies on the toxicity of surfactants in aquatic environments and microalgae are even used as model organisms to evaluate their toxicity in such ecosystems (Azizullah et al., 2014; Pane et al., 2015). As example, it is proved that biosurfactants have in general very low or inexistent toxicity and they are normally regarded to be readily biodegradable (Arutchelvi and Doble, 2011). This is the case for BSA and Saponin (Chapter 4 and Chapter 5). However, these surfactants showed at the same time a reduced potential to be used in a LF-PBR as a consequence of that. The results presented in this Thesis support that toxicity is a decisive aspect for the selection of a given surfactant, since the lack of toxicity is a mandatory requirement, but at the same time it is often negatively correlated to the microalgal partitioning and the resistant of the surfactant to biodegradation.

As described, groups of criteria to independently select microalgae and surfactants that are suitable for their application in a LF-PBR are provided in this Thesis. As a result, a microalga-surfactant combination suitable for its application in a LF-PBR was found, but the proposed criteria could be applied to select additional microalga-surfactant combinations for further research on microalgal cultivation in surfactant-stabilized foams. Nevertheless, it is advisable that the potential of the algal strains and the surfactants are evaluated jointly, using toxicity and resistance to biodegradation as benchmark for the selection.

10.3 Microalga-surfactant interactions in a LF-PBR

So far, two different LF-PBR designs have been reported, which allowed to prove the concept of microalgae cultivation in foam (Janoska et al., 2017) and to validate a mathematical model which includes several parameters involved in the LF-PBR performance, such as light penetration and gas transfer (Janoska et al., 2018a, 2018b). Based on the aforementioned model, the biomass specific energy requirement for microalgae production and harvesting was estimated to be reduced down to 8.5% of that for liquid-based cultivation. However, new designs that allow to test additional aspects of foam-bed microalgae cultivation are required. In particular, a system with a larger volumetric capacity was needed to evaluate the possible changes in the algal biomass composition derived from foam-based cultivation, which was an aim of this Thesis. These changes were expected due to possible interactions between the surfactant and the algal cells and the peculiar physicochemical conditions of the foam. In order to investigate this, a new LF-PBR was designed and constructed (Chapter 6) which is simple, easy-to-build and easy-to-operate. Such LF-PBR allowed stable microalgae cultivation in sufficient volume of liquid foam for the study of the dynamics of the algal biomass quality along foam-bed cultivation (Chapter 7). Results showed that foam-based cultivation of *Chlorella sorokiniana* in Pluronic F68-stabilized foams resulted in biomass with a distinct biochemical composition (i.e. high carbohydrate-to-protein ratio and high lutein content), suggesting that cultivation of microalgae in LF-PBRs may be used as a tool to produce algal biomass with a targeted composition. Additional microalga-surfactant combinations would open new possibilities in this sense.

Up to date, all existent LF-PBRs have been tested indoors; hence their performances have been evaluated only under controlled conditions. Due to the absence of energy consuming systems to break the foam and to recirculate the liquid, the LF-PBR described in this Thesis only requires energy to gas the system and for its illumination. Thus, the LF-PBR could be assessed outdoors and its suitability to operate using sunlight could be therefore tested with minimal energy requirements. However, it was shown that water evaporation had to be often corrected and that increased temperatures reduced the foaming capacity of the surfactant Pluronic F68 (Chapter 6). These aspects should be particularly addressed in future applications of the LF-PBR, especially outdoors.

Also, the LF-PBR hereby presented includes a three-dimensional (3D) printed piece to promote foam breakage and to redirect the liquid released. The LF-PBR is open to the environment by this piece in order to allow the release of oxygen at the top of the system but, at the same time, it results in a high evaporation rate. Another drawback of the design is the fact that the surfactant sticks to this piece. Nonetheless, thanks to the versatility of the 3D printing technology, varied designs of this crucial part of the system could be printed and assessed in order to optimize the system, while maintaining the simplicity and the low construction costs.

In summary, a simple and cheap LF-PBR is presented in this Thesis which allowed to study the changes produced in the biochemical composition of algal biomass cultivated in surfactant-stabilized foams. However, following LF-PBR designs should take into account aspects as their capacity, the materials employed and the versatility of the system in function of the purposes for which they are designed.

10.4 Further strategies in foam-based microalgae cultivation

Beyond the energy savings in cultivation and harvesting derived from cultivating microalgae in surfactant-stabilized foams, in this Thesis two additional strategies to reduce the overall production costs further have been addressed: the usage of commercial fertilizers as algal culture medium and the surfactant-mediated potential extraction of high-value compounds from the foam-based cultivated biomass.

In Chapter 8, the feasibility of a fertilizer-based culture medium in surfactant-added algal cultures was proved. The replacement of a culture medium based on refined chemicals by a medium based on a NPK (Nitrogen-Phosphorous-Potassium) fertilizer showed slightly lower biomass but high-value compounds productivities. Moreover, the usage of fertilizer-based culture media offers major advantages at large scale due to the easiness of operation and the reduced media preparation costs. In this sense, it was estimated that algal culture media preparation costs may be reduced by 3 orders of magnitude by using a fertilizer-based culture media.

However, it has to be noted that the fertilizer-based culture media tested in this Thesis was first optimized for the cultivation of *C. sorokiniana*. Then, its adequacy was assessed in combination with the surfactant Pluronic F68, since this microalga-surfactant combination was previously selected as the most promising and it was therefore used for the following studies hereby presented. Therefore, the adequacy of surfactant-added fertilizer-based culture media should be particularly assessed for additional microalga-surfactant combinations with potential to be used in a LF-PBR. Besides, the feasibility of using commercial fertilizers in the presence of a surfactant was investigated in this Thesis in liquid algal cultures as a first attempt to evaluate its feasibility in foam-based cultures. Thus, microalgae production in surfactant-stabilized foam using a fertilizer-based culture medium has still to be experimentally demonstrated and it is hereby suggested as a convenient next step on this research topic.

On the other hand, surfactants can act as extractants of valuable compounds and as lytic agents (Glembin et al., 2014; Park et al., 2014; Ulloa et al., 2012). Therefore, the possibility exists that the surfactant employed to stabilize the foam in a LF-PBR results in the extraction, at least partial, of some algal products. The potential extraction role of the surfactant can result in undesired extraction of valuable compounds during the growth phase, but it can also be regarded as a strategy to obtain valuable products while performing the foam-based cultivation process. This concept of simultaneous cultivation and extraction of valuable compounds is commonly known as "milking", and previous research have already shown it to be a cheaper alternative to harvesting, dewatering and disrupting the biomass prior the extraction process (Hejazi and Wijffels, 2004). In this Thesis, it was proved that the surfactant Pluronic F68 does not extract fatty acids from *C. sorokiniana* cells under the conditions employed along their foam-based cultivation (Chapter 9), therefore showing a good biocompatibility. However, the capability of this surfactant to extract other metabolites, such as carbohydrates or antioxidants, remains unknown. Besides, other microalga-surfactant combinations might result in the extraction of certain algal biomass products without affecting the algal cells viability. In that case, a LF-PBR could be used not only to grow microalgae and reduce the posterior harvesting effort, but its potential could be broadened by combining those processes with the extraction of targeted metabolites.

Regardless of the potential extraction role of the surfactant employed being considered as a drawback or as a tool, it should be in any case considered in the design of any foam-based microalgae cultivation process and further evaluated.

10.5 Recommendations for future research

Due to the novelty of the concept of cultivating microalgae in surfactant-stabilized foams, little research has been performed on this topic and, therefore, many aspects remain to be investigated. However, due to the high biomass productivities and expected savings in cultivation and harvesting, this cultivation concept is considered to have a large potential to overcome some of the current limitations of microalgae-based production processes. For this reason, further research on this topic seems convenient and several aspects hereby considered to be worthy of future studies are suggested below.

A critical aspect of producing microalgae in surfactant-stabilized foams is the presence of the surfactant in the biomass produced. The extent at which the surfactant should be removed from the biomass will primarily depend on its posterior application. In case of using the whole biomass for human food or animal feed, the presence of external chemicals is, in principle, undesirable. It is noteworthy that the surfactant employed in this Thesis, Pluronic F68, is approved by the Food and Drug Administration (FDA) for a variety of applications, including its use as excipient for drug delivery, as a component of adhesives used in contact with food and included in wash solutions for fruits and vegetables (PubChem Database). However, how the presence of this particular surfactant in the foam-based cultivated algal biomass would limit food and feed applications should be determined. Furthermore, from the experience obtained through this Thesis work it is also inferred that the implications regarding the safeness of the studied surfactant in human and animal consumption may differ for other surfactants.

On the other hand, for the production of highly valuable compounds, such as antioxidants, the algal cells must be broken prior to extraction and purification of those desired molecules. In this case, the final fate of the surfactant must be assessed. For instance, in this Thesis it was proved that the surfactant Pluronic F68 did not stick to *Chlorella* cells. Therefore, in the particular case of cultivating *C. sorokiniana* in Pluronic F8-stabilized foams, the biomass produced is not expected to contain large amounts of the surfactant. Nevertheless, the surfactant could interfere in the extraction process of certain metabolites while it may not be present in the final extract. In this regard, this Thesis showed that during lipid extraction the Pluronic F68 molecules ended up –at least partially- forming a layer between solvents with different polarity due to its amphipathic character (Chapter 9) which may be useful for its posterior recovery. However, this should be particularly addressed for the processes required by the final application targeted and the different surfactants.

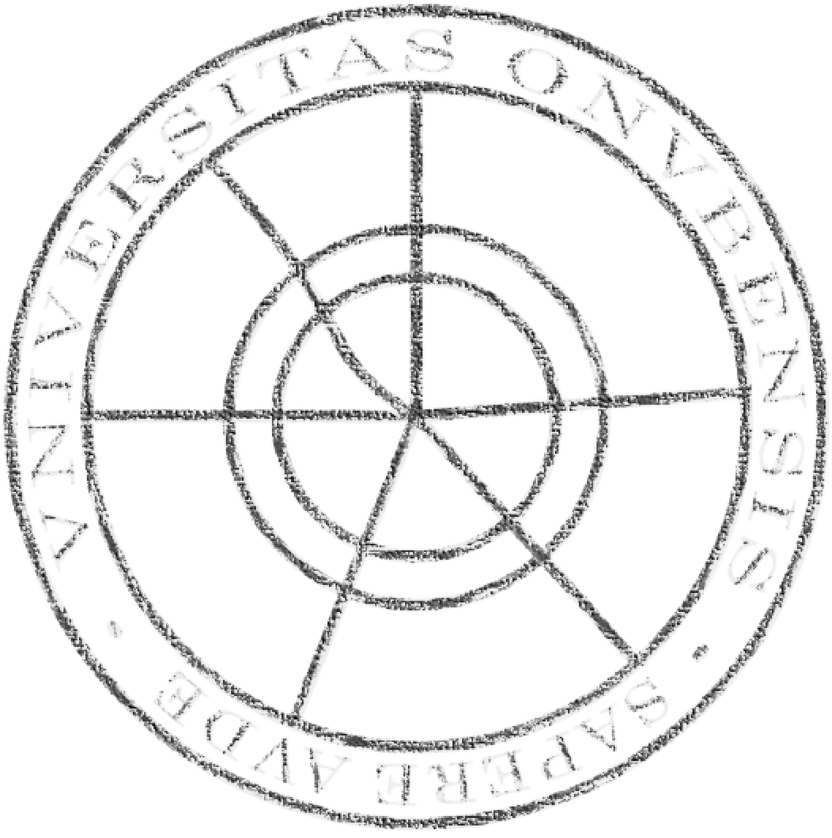
Besides, due to the expected low cost of the algal biomass produced in LF-PBR, the final purposes of the biomass would not have to be limited to the production of highly valuable compounds, but may be also employed for other purposes with lower profitability, such as biofuel production. For biofuel production, the presence of surfactant in the biomass is of little relevance as long as it does not interfere with the downstream processes. In the particular case of producing *C. sorokiniana* in Pluronic F68-stabilized foams, the algal biomass showed a large content of carbohydrates (Chapter 7), therefore it may be appropriate, for instance, for bioethanol production.

Nonetheless, regardless the final application of the biomass, the recovery of surfactant should be investigated not only to increase the final biomass quality, but also because it would be the first step towards its subsequent re-use. In this sense, the potential recycling of the surfactant would increase the sustainability of the process and lower the overall production costs.

Another relevant aspect for future research is the upscale of the foam-bed microalgae cultivation process. In the current cultivation systems, microalgae grow in liquid suspensions and research has been already performed on the interaction of the hydrodynamics, light supply, energy and mass transfer, and biological kinetics in liquid systems. Based on such existing knowledge, liquid-filled photobioreactors have been, and still are, under optimization (Pires et al., 2017; Soman and Shastri, 2015). However, several challenges would have to be faced to design and operate appropriate large scale LF-PBRs due to foam-related issues. First, increasing the reactor dimensions (particularly the reactor height) would affect foam stability. As the foam column increases, the liquid drains due to gravity and promotes foam collapse (Wang et al., 2016). Therefore, higher surfactant concentrations would be required to fill a LF-PBR of a larger volume with stable foam, which would also have implications regarding the microalgae tolerance to the surfactant. Next, it has been described that in a LF-PBR, light penetration is limited due to scattering (Janoska et al., 2018b). Thus, LF-PBR of larger volumes would result in larger dark zones and in consequence, lower biomass productivities. Light availability should therefore be considered in order to design LF-PBRs on a larger scale.

Moreover, in case of large scale cultivation in LF-PBR systems under outdoor conditions, additional issues arise. In case of an open LF-PBR design, the first challenge would be to retain the foam in it under outdoor cultivation, since wind could result in the loss of part of the foam at the top and open part of the LF-PBR. Systems to control the foam column height and appropriate reactor designs could help avoiding foam losses in open LF-PBR operated outdoors. Besides, due to the low liquid content of the foam, heat could be rapidly accumulated in a LF-PBR exposed to sunlight and water evaporation rate would increase which could also result in increased surfactant concentrations. Therefore, temperature control implementations would be of major relevance in order to avoid microalgal growth inhibition or even cell death due to excessive temperatures in the reactor.

To summarize, this Thesis attempted to provide insight on several aspects of microalgae cultivation in surfactant-stabilized foam, and based on the scientific information obtained and discussed it can be stated that much research has still to be performed in order to achieve successful microalgae cultivation in LF-PBRs on an industrial scale. Nonetheless, the energy savings offered and the still unraveled full potential of this cultivation concept make it a promising tool for cost-effective microalgae-based production processes.



IV. CONCLUSIONS

Conclusiones

Chapter 3

El potencial de una cepa de microalga para ser cultivada en espumas estabilizadas por un surfactante puede ser evaluado de acuerdo a los siguientes criterios: alta/moderada espumabilidad, enriquecimiento de la espuma en microalgas y estabilización de la espuma por parte de la suspensión algal y crecimiento rápido y robusto de las células algales. La adición de un surfactante es considerada necesaria para ela evaluación de estos parámetros, así como para el cultivo de microalgas en un fotobiorreactor de espumas líquidas (LF-PBR). La aplicación de estos criterios de selección a 6 cepas de microalga usando BSA como surfactante permitió la selección de *Chlorella sorokiniana*, *Nannochloropsis gaditana* y *Scenedesmus obliquus* como cepas prometedoras para ser producidas en espumas estabilizadas por surfactante.

Chapter 4

El potencial de un surfactante para ser empleado en un fotobiorreactor de espumas líquidas puede ser evaluado de acuerdo a sus propiedades espumantes, su biodegradabilidad y su toxicidad para las microalgas, así como al enriquecimiento de la espuma en microalgas. La aplicación de estos criterios de selección usando las microalgas previamente seleccionadas permitieron identificar a lo surfactantes poloxámeros, en particular el Pluronic F68, como surfactantes prometedores para la producción de microalgas en espumas estabilizadas por surfactantes.

Chapter 5

El uso de surfactantes que promueven el crecimiento de bacterias al ser utilizados como fuente de carbono y energía resulta en la disminución de la capacidad espumante de los cultivos y de su potencial para ser utilizados en un LF-PBR. Por tanto, los surfactantes biodegradables son menos adecuados para la producción de microalgas en espumas estabilizadas por surfactantes.

Chapter 6

Se diseñó, construyó y puso en marcha un LF-PBR adecuado para evaluar la producción de microalgas en espumas estabilizadas por surfactantes a escala de laboratorio. El LF-PBR permite la producción de espuma, su rotura mediante una pieza impresa en 3 dimensiones y la recirculación del líquido de forma continua y con bajos requerimientos energéticos. La termosensibilidad del surfactante, la correción de la evaporación y el descenso de la concentración de surfactante debido a su pegado al sistema son aspectos dignos de mencionar con respecto a la operación del LF-PBR durante períodos prolongados.

Chapter 7

El empleo de surfactantes adecuados en concentraciones apropiadas para el cultivo de microalgas en espumas estabilizadas por surfactantes en un LF-PBR resulta en una mejora del crecimiento de las microalgas y del consumo de nutrientes con respecto a cultivos líquidos. Las características físico-químicas del cultivo en espuma (p.e. alta disponibilidad de CO_2) y/o las posibles interacciones entre el surfactante y la superficie de las células de mciroalga parecen ser factores clave que explican ese comportamiento. El cultivo en espuma podría desencadenar el estrés intensificado y los cambios en el perfi bioquímico observado en microalgas producidas en el LF-PB.

Chapter 8

El uso de fertilizantes NPK en combinación con el surfactante Pluronic F68 o con el tiempo otros surfactantes poloxámeros, puede ser una estrategia prometedora para la producción sostenible de microalgas en espuma. Esto se demostró en cultivos líquidos de *C. sorokiniana*, en términos productividad de biomasa y de luteína. Además del efecto en el crecimiento, en el escalado de la producción de microalgas con fertilizantes NPK se debe prestar especial atención a la concentración total de fósforo proporcionada, ya que uega un papel en el tamponamiento del pH del cultivo.

Chapter 9

El Pluronic F68 no compromete la integridad de las células de *C. sorokiniana* ni promueve la extracción de ácidos grasos durante el cultivo ni tras la incubación con el surfactante a concentraciones hasta 10 veces la usada e el LF-PBR. Por tanto, se puede concluir que el Pluronic F68, o eventualmente otros surfactantes poloxámeros, es biocompatible con *C. sorokiniana.*

Como conclusión general de esta Tesis, se puede afirmar que el cultivo en espumas estabilizadas por surfactante es un concepto novedoso y prometedor para la producción rentable de biomasa de microalgas y sus productos derivados, aunque deben realizarse más estudios para maximizar su potencial.

Conclusions

Chapter 3

The potential of a microalgal strain to be cultivated in surfactant-stabilized foams can be evaluated according to the following criteria: high/moderate foamability, algal partitioning and foam stabilization by the algal suspensions, and high robustness and fast growth of the algal cells. The addition of a surfactant is deemed necessary for the assessment of these parameters, as well as for microalgae cultivation in a liquid foam-bed photobioreactor (LF-PBR). The application of these selection criteria to 6 algal strains using BSA as surfactant allowed the selection of *Chlorella sorokiniana*, *Nannochloropsis gaditana* and *Scenedesmus obliquus* as promising strains to be produced in surfactant-stabilized foams.

Chapter 4

The potential of a surfactant to be employed in a liquid foam-bed photobioreactor can be evaluated according to its foaming properties, its biodegradability, its toxicity to microalgae, and the microalgal partitioning towards the foam. The application of these criteria using the algal strains previously selected allowed to identify poloxameric surfactants, in particular Pluronic F68, as promising surfactants for algae production in surfactant-stabilized foams.

Chapter 5

The use of surfactants that promote bacterial growth on their carbon and energy content results in decreased foaming capacity of the cultures and reduced potential to be used in a LF-PBR. Thus, biodegradable surfactants are less suitable for algae production in surfactant-stabilized foams.

Chapter 6

A LF-PBR suitable to test microalgae production in surfactant-stabilized foams at lab-scale was designed, built and set up. The LF-PBR allows continuous foam production, foam breakage in a three-dimensional printed top piece, and liquid recirculation with low energy requirements. Surfactant thermosensivity, correction of water evaporation and surfactant concentration decrease due the surfactant sticking to the system are noteworthy aspects for long-term operation of the LF-PBR.

Chapter 7

The usage of suitable surfactants and concentrations for cultivation of microalgae in stabilized foams in LF-PBR results in improved microalgal growth and nutrients consumption compared to liquid cultures. The physicochemical characteristics of the foam-bed cultivation (e.g. high CO_2 availability) and/or the possible interactions between the surfactant and the algal cell surface seem to be key features to explain that behavior. Cultivation in foam might trigger the intensified stress and shifted biochemical profile observed in microalgae produced in LF-PBR.

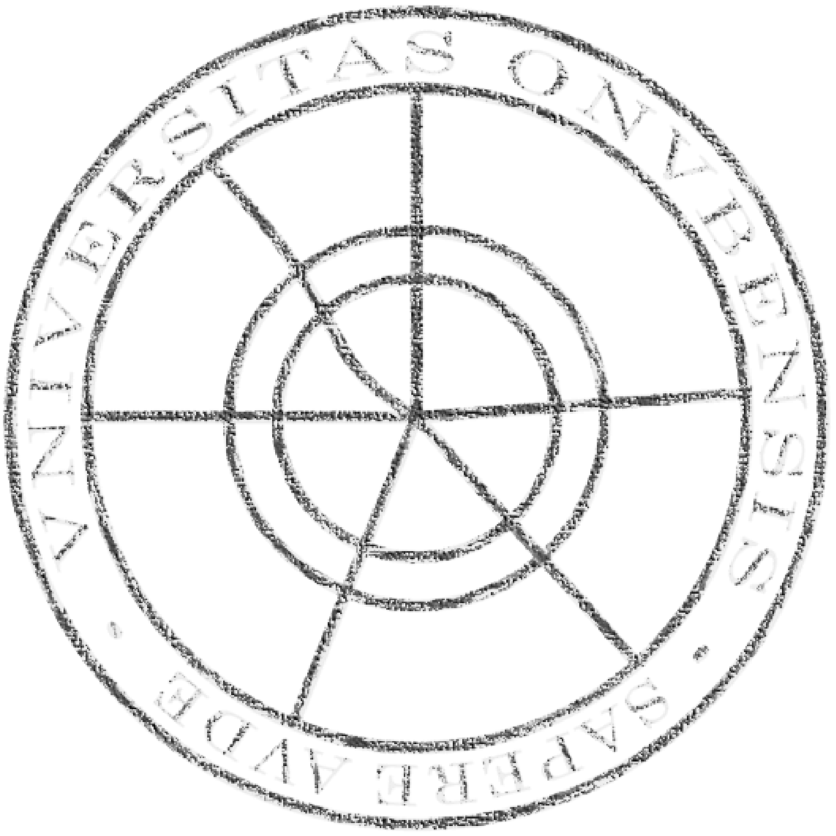
Chapter 8

The use of NPK fertilizers in combination with the surfactant Pluronic F68, or eventually other poloxameric surfactants, can be a promising tool for sustainable production of microalgae in foam. This was proved in liquid cultures of *C. sorokiniana*, in terms of biomass and lutein productivities. Besides their effect in growth, in the scale up of NPK fertilizers-based microalgal production, special attention should be paid at the total phosphorous concentration provided since it plays a role in buffering the culture pH.

Chapter 9

Pluronic F68 does not compromise the integrity of *C. sorokiniana* cells or promote the extraction of fatty acids during cultivation or by incubation with the surfactant at concentrations up to 10-fold higher than the one used in the LF-PBR. Thus, it can be concluded that Pluronic F68, or eventually other poloxameric surfactants, is biocompatible with *C. sorokiniana.*

As the main conclusion arosen from this Thesis, it can be stated that cultivation in surfactant-stabilized foams is a promising novel concept for cost-effective production of microalgal biomass and derived products, but further research should be carried out in order to maximize its potential.



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About the author

María Vázquez Toscano was born on May 23rd 1987 in Huelva, Spain. She started her studies on Biotechnology in 2005 at University of León, Spain. In 2009 she obtained a Séneca scholarship and she moved to Seville for a year to conduct her studies at Pablo de Olavide University. During her studies she got an internship at the Microbiology department of University of León and she did another internship and worked as a microbiology analyst in Lacoal S.L., a laboratory dedicated to assess the quality of food and water. This allowed her to gain lab experience in molecular biology and microbiology.

After finishing her BSc studies, she started collaborating in the Algal Biotechnology group (BITAL) at University of Huelva. She got involved in a research project within BITAL group in collaboration with the company Fertiberia S.A., in which she studied the potential of agricultural fertilizers for microalgae production. She did a 6-month internship (Marie Curie programme) at La Frontera University (Temuco, Chile) and she studied microalgal cell synchronization as a tool to improve its anaerobic digestion for biogas production. In 2013 she started her MSc studies in Chemistry at University of Huelva and in 2014 she started her PhD studies within the BITAL group by getting involved in MIRACLES project (financially supported by the UE under the 7th Framework Programme). Her PhD Thesis focused on the novel concept of microalgae production in surfactant-stabilized foams in a liquid foam-bed photobioreactor. During her PhD she did a 3-month internship at Wageningen University, since her PhD project was performed in a close collaboration with this University.

Since September 2018 she is working within the BITAL group at the University of Huelva. She is involved in a research project in collaboration with the companies Algenion GmbH (Germany) and Ad Astra Ehf (Iceland). Within this project she is testing a novel microalgae cultivation system and optimizing astaxanthin production from *Haematococcus pluvialis.*

List of publications

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** Contributed equally to this work*

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