Integrated Master in Bioengineering

Mass balance analysis of carbon and nitrogen in industrial mixotrophic microalgae cultures

Dissertation for Master Degree in Bioengineering

Specialization in Biological Engineering

Faculdade de Engenharia da Universidade do Porto – Departamento de Engenharia Química

Ana Isabel Faustino Barros

Porto, September 2014

Coordination: (A4F – AlgaFuel, S.A.) Luís Costa, Ph. D.

Tiago Guerra, Ph. D.

(FEUP) Manuel Simões. Ph. D.

“First you make it then you master it

and after that you matter.”

Raymond John

Acknowledgements

This work would not have been possible without the advice and support of many people.

First, I would like to acknowledge A4F-AlgaFuel, S.A. for the funding and above all for the outstanding opportunity that this study represented. Furthermost I must very much thank my advisors Luís Costa and Tiago Guerra for the time and dedication that was put into all the analytic discussions which were fundamental to enlighten all the metabolic and process aspects of this study. I am also extremely grateful to Professor Manuel Simões for his role in my academic course and especially for introducing and accompany me in the microalgae world.

I would also like to thank all AlgaFuel staff that in a very friendly manner helped me with the day to day tasks of the work and to those that distracted me when distraction was needed.

Finally, in the lame part of things, I absolutely need to thank my family (dog included) for the patience and support, Samuel for his never-ending well of confidence in me, my friends for providing me with the best times of my life in Porto and to everyone that played a part in who I am today.

Abstract

The large-scale cultivation of *Chlorella vulgaris* has great interest given the amount of products and potential applications that can derive from its biomass. From an industrial point of view, it is necessary to rapidly obtain high density cell cultures in order to increase productivity at lower production costs. The fundamental mass balance of carbon and nitrogen is therefore necessary to quantify the recovery and consumption yields of these nutrients in the global process of biomass production.

In an industrial microalgae production facility in Portugal – Algafarm – recovery factors of 0.94 ± 0.01 and 0.76 ± 0.30 were obtained for carbon and nitrogen mass balance, respectively. Mixotrophic growth of *C. vulgaris* with acetate and urea as carbon and nitrogen sources, respectively, yielded an overall productivity of 0.087 g L^{-1} day^{-1} with a maximum growth rate of 0.673 d^{-1}. The acetate yield obtained, 0.67 g_X g_{HAc}^{-1}, is close to the theoretical yield of 0.8 g_X g_{HAc}^{-1}in opposition with the urea yield of 3.61 g_X g_{Urea}^{-1}which is lower than its theoretical value of 4.67 g_X g_{Urea}^{-1}. Furthermore, global carbon and nitrogen yields of 0.76 $mol_{C\text{-}X}$ mol_C^{-1} and 0.72 $mol_{N\text{-}X}$ mol_N^{-1} were determined.

Results indicate that the carbon atoms of acetate and urea are incorporated into the biomass. Therefore inorganic carbon dioxide was concluded to have little influence on microalgal cultivation in the conditions studied, apart from pH control. Furthermore, urea metabolism results in ammonium build-up in the culture medium. Nonetheless, the satisfactory yields obtained indicate that urea and ammonium are effectively used by *C. vulgaris* cells. These high carbon and nitrogen yields resulted, however, in low biomass growth. Indeed, this study pointed to an imbalance in the nutrient proportions of the feed that led to improvements to the production process. Additionally, the production could be undergoing close to starvation. So, further study is needed to enlighten nutrient utilization by the cells. Namely, nutrient distribution throughout the photobioreactors and biomass biochemical composition along the scale-up are subjects that require more detailed attention. This would enable the alignment of feeding streams with metabolic needs that should reflect in higher biomass productivities.

Key-words: *Chlorella vulgaris*, mixotrophic metabolism, acetate, urea, biomass productivity.

Resumo

A produção de *Chlorella vulgaris* em larga-escala é de grande interesse dadas os atuais e potenciais aplicações da sua biomassa. Contudo, do ponto de vista industrial, a obtenção rápida de culturas com uma elevada concentração celular é fundamental tendo em vista o aumento da produtividade e a diminuição dos custos de produção. Desta forma, o balanço mássico ao carbono e azoto é necessário para avaliar a eficiência da incorporação dos nutrientes na biomassa produzida.

Numa unidade de produção de microalgas portuguesa, a Algafarm, foi então possível obter factores de recuperação do carbono e azoto de 0.94 ± 0.01 e 0.76 ± 0.30 respetivamente. O cultivo mixotrófico de *C. vulgaris* resultou numa produtividade de 0.087 g L^{-1} dia^{1} com uma taxa específica máxima de crescimento de 0.673 d^{-1}. Foi obtido um rendimento global em acetato de 0.67 g_X g_{HAc}^{-1}, próximo do seu valor máximo teórico de 0.8 g_X g_{HAc}^{-1}. Por outro lado, o rendimento global obtido para a ureia, de 3.61 g_X g_{Urea}^{-1}, revelou-se aquém do seu valor teórico de 4.67 g_X g_{Urea}^{-1}. Os rendimentos globais de incorporação do carbono e do azoto na biomassa foram determinados resultando num valor de 0.76 $mol_{C\text{-}X}$ mol_C^{-1} e 0.72 $mol_{N\text{-}X}$ mol_N^{-1}.

Os resultados obtidos indicam que os átomos de carbono presentes nas moléculas de acetato e ureia são incorporados na biomassa. Desta forma é possível concluir que, nas condições do estudo, a injecção de dióxido de carbono inorgânico no meio de cultura reflecte-se sobretudo na diminuição o valor do pH. Por outro lado, apesar de o metabolismo da ureia resultar no aparecimento do ião amónio no meio de cultura, o elevado rendimento de incorporação do azoto na biomassa é indicativo de que a ureia e o amónio são eficazmente utilizados pelas células. Contudo, estas elevadas eficiências de incorporação de C e N na biomassa não se traduziram numa elevada produtividade do sistema. Tal facto permitiu diagnosticar deficiências na composição do meio de cultura. Não foi contudo possível esclarecer se as células são submetidas a condições de fome durante o cultivo sendo ainda necessária a realização de um estudo mais aprofundado da utilização dos nutrientes pelas células ao longo do fluxo nos fotobiorreatores e ao longo do processo de *scale-up* em geral. Em teoria isto permitiria a obtenção de maiores produtividades através da adequação da alimentação com as necessidades metabólicas das células.

Palavras-chave: Chlorella vulgaris, metabolismo mixotrófico, acetato, ureia, produtividade.

Table of Contents

List of Tables

List of Figures

Nomenclature

Abbreviations

CMP	Cimentos Maceira e Pataias, S.A.
CoA	coenzyme A
DCMU	1-(3,4-dichlorophenyl)-3,3-dimethylurea
GS/GOGAT	glutamine synthase/ glutamine oxoglutarate aminotransferase
PBR	photobioreactor
TCA	tricarboxylic acid

Indexes

a	afternoon (19 h)
bas	basal medium streamstream
feed	feeding medium streamstream
harv	harvesting stream
I	incident light
i	indicator of time (day)
ino	inorganic
m	morning (9 h)
PBR	photobioreactor
res	respiration stream
S	organic substrate
sup	supplementation stream
X	biomass

Measuring units

μ	specific growth rate	h^{-1}
μ_{max}	maximum specific growth rate	h^{-1}
Am	ammonium ion concentration	mol L^{-1}
C:N	carbon to nitrogen ratio	$mol_C\ mol_N^{-1}$
DW	dry weight	g L^{-1}
f_C	carbon conversion factor	$mol_C\ g_X$
f_N	nitrogen conversion factor	$mol_N\ g_X$
HAc	acetate/acetic acid concentration	mol L^{-1}
$h\upsilon$	radiation energy	
I	incident light	W m^{-2}
K_α	saturation constant of substance α	g L^{-1}
Nit	nitrate concentration	mol L^{-1}
pK_α	negative $\log_{10}$ of the acid dissociation constant of a solution.	
P_X	biomass productivity	$kg_X\ L^{-1}\ d^{-1}$
S	organic substrate concentration	g L^{-1}
Ur	urea concentration	mol L^{-1}
V	volume	m^3
X	biomass concentration	g L^{-1}
$Y_{X/\alpha}$	yield of biomass on substance α	$g_X\ g_\alpha^{-1}$ or $mol_X\ mol_\alpha^{-1}$

Chapter 1

1. Work outline

1.1. Background and project presentation

Microalgae cultures have been widely studied given the vast variety of commercial applications of their biomass and metabolites (Spolaore et al. 2006; Molina Grima et al. 2003; Borowitzka 1999; Perez-Garcia et al. 2011). However, achieving high density cultures is not easy (Choi et al. 2012; Abreu et al. 2012). Presently the most common method for the cultivation of microalgae is by autotrophic growth (Safi et al. 2014; Kim et al. 2013; Perez-Garcia et al. 2011; Yeh et al. 2012). In this way microalgae grow photoautotrophically using carbon dioxide as the carbon source and light as the energy source (Neilson & Lewin 1974). Thus, the presence of sufficient light presents a major limitation to high biomass productivities. This extreme dependence on local weather conditions makes the production seasonal (Rawat et al. 2013; Yang et al. 2000; Perez-Garcia et al. 2011; Safi et al. 2014; Chisti 2007). Hence, in order to overcome these autotrophic limitations and to increase productivity mixotrophic growth was implemented at the Algafarm production unit, a joint project for microalgae production on industrial scale in Portugal by Secil/CMP and A4F. Mixotrophy is commonly defined as the sum between photoautotrophic and heterotrophic metabolism as it uses light energy along with an organic source of carbon. Mixotrophic growth has been reported to enhance biomass productivity up to 5.5 fold with the additional advantage of maintaining the production of photosynthetic metabolites (Perez-Garcia et al. 2011). Understanding nutrient utilization by microalgae cultures is crucial to develop the high cellular density culture systems necessary for the economic feasibility of the biomass production (Scherholz & Curtis 2013). Thereby the mass balance of key nutrients is important to optimize microalgae cultivation as it enables the assessment of individual and global yields. As laboratory small scale systems do not accurately describe the complex industrial cultivation processes, it is necessary to construct and validate the mass balance of the large scale systems incorporating the specific geographic and biological characteristics of the production location (Quinn et al. 2011).

1.2. Main objectives

The mass balance of a system allows the evaluation of its efficiency of transforming input feeds to output products. Mass balances are important for any industry as the control of processing and of global yields is fundamental. In order to do so successfully, it is necessary to understand the system in detail. This is of greater importance in biological systems given their complex operational challenges related to the highly-adaptable nature of the cell's metabolism. Therefore, the objectives proposed for this project were to:

1. Characterize all input and output fluxes of the system in terms of carbon and nitrogen;
2. Monitor biomass growth and nutrient levels in the photobioreactor;
3. Determine the specific growth rate and biomass productivity throughout the scale-up process in the specific conditions of the study;
4. Determine the yield of the carbon and nitrogen substrates throughout the scale-up process in the specific conditions of the study;
5. Determine the carbon to nitrogen ratio of the feeding streams and consumed by the biomass throughout the scale-up process in the specific conditions of the study;
6. Estimate the elemental composition of the biomass produced;
7. Determine the incorporation of every substrate feed into the biomass produced;
8. Implement, at the industrial unit, a simple and reliable method for routine mass balance quantification;

Finally with this information it will possible to re-evaluate the need for carbon and nitrogen nutrient supply and the subsequent optimization of the feed.

1.3. Thesis organization

The present study is divided in six chapters. The present Chapter, one, aims to present the motivations and objectives of the project.

Chapter 2 consists in a short literature review on the main cultivation methods of microalgae with focus to the possible trophic states that *Chlorella vulgaris* can grow on.

The goal of this review is to enlighten metabolic use of the substrates fed to the cultures. Hence acetate and urea assimilation pathways in such conditions are described in detail.

Chapter 3 is a basic overview of the Algafarm production system comprising the scale-up of the cultures and the design and operation of its photobioreactors for better understanding of the subsequent mass balance.

Chapter 4 describes the study of the consumption of nutrients by *Chlorella vulgaris* cells grown mixotrophically. Growth productivities and yields on acetate, urea, carbon and nitrogen are presented. Elemental composition of the biomass is also discussed in order to determine the carbon to nitrogen ratio incorporated as compared to that of the feed.

Chapter 5 comprises the assumptions and methods applied in the mass balance analysis. The fit of the model is evaluated along with the analysis of its sources of error.

Lastly, Chapter 6 presents the general conclusions of the study as well as the identified needs for improvement and future work.

Chapter 2

2. Literature review

2.1. *Chlorella* spp. cultures: cultivation methods and metabolism

Large-scale microalgae production has been considered since the 1950's given their high potential as cell-factories (Chisti 2007; Spolaore et al. 2006). *Chlorella* spp. applications range from bioremediation to high-value biochemicals (Spolaore et al. 2006; Safi et al. 2014). Potential and actual products such as carbohydrates, lipids, pigments, minerals, vitamins, enzymes, polymers and toxins can be obtained by the cultivation of microalgae in different mineral media, with organic substrates or even in wastewater (Spolaore et al. 2006; Perez-Garcia et al. 2011; Safi et al. 2014). The culture medium must be selected in accordance with the intended application for the *Chlorella* spp. biomass as their biochemical composition is altered by changes in media composition and/or trophic conditions (Chia et al. 2013; Safi et al. 2014; Kong et al. 2013; Crofcheck et al. 2013; Mandalam & Palsson 1998; Chojnacka & Marquez-Rocha 2004; Lin 2005). Generally stress conditions caused by nutrient depletion, excess of illumination among others, result in the accumulation of lipids and/or starch by *Chlorella* spp. cells hindering biomass productivity (Courchesne et al. 2009; Safi et al. 2014; Lin 2005). On the opposite, protein content increases in normal growth conditions where all nutrients are available and the operating conditions such as temperature and light exposure are favourable for biomass productivity (Safi et al. 2014). Accordingly, mixotrophically grown *Chlorella* spp. cells have been shown to possess suitable qualities for use as health foods (Lin 2005). In a similar way, heterotrophically grown algal cells have high digestibility and biological value as feedstock for animals and fish (Chojnacka & Marquez-Rocha 2004).

Microalgae mass production has been achieved to date mainly in open raceway ponds and tubular or flat plate photobioreactors with optimization of photoautotrophy as the main goal (Safi et al. 2014; Chisti 2007; Harun et al. 2010; Grima et al. 1999; Lee 2001; Borowitzka 1999). Open raceway ponds, despite being a cheap and easy to scale-

up installation for the production of microalgal biomass, are extremely dependent on local climate conditions and present significant difficulties in the control of nutrients and external contamination (Harun et al. 2010; Perez-Garcia et al. 2011; Safi et al. 2014; Chisti 2007). Thereby, pharmaceutical and food applications are very limited or impossible with this production method. In order to reduce contamination problems, open systems can be operated in high salinity or extreme pH limiting its application to extremophile species (Harun et al. 2010; Perez-Garcia et al. 2011; Lee 2001; Borowitzka 1999). On the other hand, closed photobioreactors allow the operation in mild conditions with better control of contamination enabling the cultivation of a wider variety of species (Borowitzka 1999; Lee 2001; Harun et al. 2010; Safi et al. 2014; Chisti 2007). The controlled environment allows the operation in continuous or semi-continuous modes enhancing the biomass volumetric productivity up to 13 times in comparison with raceway ponds (Lee 2001; Harun et al. 2010; Safi et al. 2014; Chisti 2007; Borowitzka 1999) although the effect on the biomass areal productivity should also be considered in a global analysis. Consequently, the harvesting costs are lowered by more concentrated cultures and the quality of the product becomes more consistent throughout the batches (Borowitzka 1999). Additionally, closed cultivation systems allow a higher light availability and facilitate gas exchanges (Borowitzka 1999). Nevertheless, the high illuminated surface area to volume needed for successful cultivation precludes heat sterilization of the system unless high investment is applied. Consequently, disinfection is achieved by chemical agents that do not completely remove bacterial contamination thus not fulfilling the requirements for pharmaceutical products (Lee 2001). In this way, commercialization of microalgal products, using these systems, is limited to food and feed products of medium volume and value. The initial investment and the capital and energy demand for maintaining mass transfer and cell suspension in mild conditions with this type of equipment are very high and so the biomass productivities must be largely increased to offset production costs.

The use of closed systems enables microalgae growth in photoautotrophic, mixotrophic or heterotrophic conditions (Borowitzka 1999). When grown heterotrophically on sugar or organic acids in the dark or mixotrophically using light energy and organic carbon/energy concurrently, different production systems can be applied (Lee 2001; Perez-Garcia et al. 2011; Borowitzka 1999). For heterotrophic growth any basic stirred tank reactor can be used for biomass mass production reducing

development and operational costs (Perez-Garcia et al. 2011; Lee 2001; Borowitzka 1999). On the other hand, for mixotrophic growth a photosynthetic phase in the bioreactor must be maintained as light remains necessary as an energy source and for induction of the needed phototrophic metabolic apparatus (Lee 2001). Thus, closed photobioreactors or a two-stage photosynthetic/dark system are the most suitable system for the mixotrophic growth of microalgae. However, large-scale mixotrophic axenic cultures are impossible to obtain as the sterilization problems persist and contamination development is enhanced by the presence of organic carbon substrates (Lee 2001). For mixotrophic cultures the organic substrate must then be gradually supplemented to the culture during illumination periods and sometimes stopped some time before sunset in order to preclude bacterial proliferation at night (Lin 2005; Chisti 2013; Lee 2001).

2.2. Microalgae metabolism

Microalgae present enormous biodiversity with more than 40 000 species described (Safi et al. 2014). Specifically, *Chlorella vulgaris* is a fast-growth green eukaryote with asexual reproduction (Safi et al. 2014; Lin 2005; Liang et al. 2009). Furthermore it can proliferate through various trophic states: photoautotrophy, heterotrophy, mixotrophy and photoheterotrophy (Lee 2001; Yang et al. 2000; Kim et al. 2013; Perez-Garcia et al. 2011; Muthuraj et al. 2013; Yeh & Chang 2012; Borowitzka 1999; Martinez & Orus 1991; Yang et al. 2011; Chia et al. 2013; Liang et al. 2009; Lin & Chen 1994; Kong et al. 2013; Scherholz & Curtis 2013; Sforza et al. 2012; Neilson & Lewin 1974; Lin 2005; Chojnacka & Marquez-Rocha 2004; Ogawa & Aiba 1981). In spite of the great differences in the cultures growth rate (Table 2.2.1), cytologically there seems to be no difference between autotrophic and mixotrophically grown cells in opposition with the absence of green pigments in heterotrophic cultures (Lin & Chen 1994), but this can change according to the species in question.

Table 2.2.1 – Maximum growth rates of *Chlorella* spp. under different growth conditions and trophic states (photoautotrophy, heterotrophy, mixotrophy and photoheterotrophy).

Trophic state	Species	Maximum specific growth rate (d^{-1})	Growth medium	Carbon source	Nitrogen source	Working volume (L)	Light:Dark cycle	Reference
photo auto	*Chlorella vulgaris*	0.840	LC oligo	CO_2	NO_3	0.2	16:8	(Chia et al. 2013)
	Chlorella sp.	0.035	F/2	CO_2	NO_3	14	12:12	(Choi et al. 2012)
	Chlorella vulgaris	0.192	-	CO_2	urea	0.4	16:8	(Crofcheck et al. 2013)
	Chlorella vulgaris	1.920	Bristol 3N	CO_2	NO_3	9.6	24:0	(Filali et al. 2011)
	Chlorella sorokiniana	0.240	PM	CO_2	NO_3	0.6	24:0	(Kim et al. 2013)
	Chlorella vulgaris	0.480	SEM	CO_2	NO_3	0.1	12:12	(Kong et al. 2013)
	Chlorella vulgaris	0.010	-	CO_2	NO_3	1.0	24:0	(Liang et al. 2009)
	Chlorella vulgaris	1.950	Rodriguez-Lopez A	CO_2	NO_3	0.5	24:0	(Martinez & Orus 1991)
	Chlorella sp.	1.080	BG-11	CO_2	NO_3	3.0	16:8	(Muthuraj et al. 2013)
	Chlorella vulgaris	0	MC + DCMU	CO_2	NO_3	0.1	24:0	(Ogawa & Aiba 1981)
		2.640	MC	CO_2	NO_3	0.1	24:0	
	Chlorella sorokiniana	1.500	SK mineral salt	CO_2	NO_3	0.5	24:0	(Pollo 2012)
	Chlorella	0.300	BG-11	CO_2	peptone	0.25	24:0	(Sforza et al. 2012)

	protothecoides							
hetero	*Chlorella sorokiniana*	0.530	PM	glucose (5 g L^{-1})	NO_3	0.6	0:24	(Kim et al. 2013)
	Chlorella vulgaris	1.200	Rodriguez-Lopez A	glucose (5 g L^{-1})	NO_3	0.5	0:24	(Martinez & Orus 1991)
	Chlorella sp.	1.296	BG-11	glucose (15 g L^{-1})	NO_3	3.0	0:24	(Muthuraj et al. 2013)
	Chlorella vulgaris	2.640	MC + DCMU	glucose (5g L^{-1})	NO_3	0.1	0:24	(Ogawa & Aiba 1981)
		2.352	MC	glucose (5 g L^{-1})	NO_3	0.1	0:24	
	Chlorella protothecoides	0.870	BG-11	glycerol (1%)	peptone	0.25	0:24	(Sforza et al. 2012)
mixo	*Chlorella* sp.	0.076	F/2	glucose (1 g L^{-1})	NO_3	14	12:12	(Choi et al. 2012)
	Chlorella sorokiniana	0.440	PM	glucose (2.5 g L^{-1})	NO_3	0.6	24:0	(Kim et al. 2013)
	Chlorella vulgaris	0.990	SEM	glucose (2 g L^{-1}) + glycerol (5g L^{-1})	NO_3	0.1	12:12	(Kong et al. 2013)
	Chlorella vulgaris	0.254	-	glucose (1%)	NO_3	1.0	24:0	(Liang et al. 2009)
		0.087	-	glycerol (1%)	NO_3	1.0	24:0	
		0.102	-	acetate (1%)	NO_3	1.0	24:0	
	Chlorella vulgaris	3.160	Rodriguez-Lopez A	glucose (5 g L^{-1}) + CO_2 (2%)	NO_3	0.5	24:0	(Martinez & Orus 1991)
	Chlorella vulgaris	2.712	MC + DCMU	glucose (5g L^{-1})	NO_3	0.1	0:24	(Ogawa & Aiba 1981)

		4.752	MC	glucose (5 g L^{-1})	NO_3	0.1	0:24	
	Chlorella sorokiniana	1.600	SK mineral salt	glucose (0.6 g L^{-1}) + CO_2	NO_3	0.5	24:0	(Pollo 2012)
	Chlorella protothecoides	0.910	BG-11	glycerol (1%)	peptone	0.25	0:24	(Sforza et al. 2012)
		1.450	BG-11	acetate (0.8%) + CO_2	peptone	0.25	0:24	
	Chlorella vulgaris	0.334	OCM	glucose (15 g L^{-1})	yeast extract	0.1	-	(Heredia-Arroyo et al. 2011)
		0.333	OCM	glycerol (20.4 g L^{-1})	yeast extract	0.1	-	
		0.028	OCM	acetate (20.5 g L^{-1})	yeast extract	0.1	-	
photo hetero	*Chlorella minutissima*	0.408	-	glycerine (25.2 g L^{-1})	casein	1.5	24:0	(Yang et al. 2011)

2.2.1. Photoautotrophic growth

Photoautotrophy implies the use of light as the energy source for the oxidation of water (H_2O) to oxygen (O_2) coupled with the fixation/reduction of carbon dioxide (CO_2) to sugars for cell use (1) (Chojnacka & Marquez-Rocha 2004).

$$aHCO_3^- + bCH_4N_2O + cH_2O \xrightarrow{kcal_{hv}} C_qNH_pO_n + \alpha O_2 + \beta OH^- \quad (1)$$

As microalgae grow in aqueous solutions, carbon dioxide is dissolved as a part of a weak acid-base buffer system. When pH ranges between 6.5 and 10, the dominant form of this system is bicarbonate as presented on the above equation. In this case, the general formula $C_qNH_pO_n$ represents biomass and urea is presented as the nitrogen source for demonstration purposes and can be substituted by any other suitable molecule. From the equation one can easily conclude that photoautotrophic growth results in pH increase.

In photoautotrophic cultures biomass growth is closely related to photosynthetic activity and the growth limiting substrates are light and CO_2 concentration. The influence of light in *Chlorella vulgaris* growth is often described by Monod kinetics (2) since there is usually no photoinhibition and can be generally described by the following relations (Chojnacka & Marquez-Rocha 2004):

$$\mu = \mu_{max}\left(\frac{I}{I+K_I}\right) \quad (2)$$

- $I < K_I$: the growth rate is linear to the incident light that represents the limiting factor of growth.
- $I > K_I$: the specific growth rate is no longer dependent on incident light but is limited by other environmental or physiological conditions, for instances CO_2 concentration or saturation of the photosynthetic apparatus, respectively.

As previously stated, this trophic regime is the most common metabolic condition applied for microalgae cultivation (Chen et al. 2011). However, light penetration is inversely proportional to biomass concentration due to the severe mutual cell shading effect attained in such conditions. High productivities are thus not possible resulting in higher harvesting costs (Liang et al. 2009). On the other hand, the use of sunlight and

atmospheric CO_2, natural and inexpensive resources, is a huge industrial advantage in favourable weather conditions (Liang et al. 2009; Chen et al. 2011). Furthermore, it can contribute to global carbon dioxide reduction, presently a matter receiving global attention, and reduce the contamination problem of microalgae cultures (Chen et al. 2011).

2.2.2. Heterotrophic growth

Heterotrophy in microalgae refers to the capability of sustained growth and cell division in the dark when both energy and carbon sources are acquired exclusively from an organic substrate (Neilson & Lewin 1974). This metabolic condition is also commonly referred to as respiration. Respiration pathways are common to all microorganisms and comprise the oxidation of an organic substrate to CO_2 with consumption of molecular oxygen with the aim of providing carbon skeletons and energy for biosynthesis and maintenance (3) following simple Monod kinetics (4) (Chojnacka & Marquez-Rocha 2004).

$$dCH_iO_m^- + bCH_4N_2O + eO_2 \xrightarrow{kcal_{CH_iO_m}} C_qNH_pO_n + \varepsilon H_2O + \Phi HCO_3^- + \zeta H^+ \quad (3)$$

$$\mu = \left(\mu_{max,S}\frac{S}{S+K_S}\right) \quad (4)$$

Again urea is presented as the nitrogen source for demonstration purposes and can be substituted by any other suitable molecule, $C_qNH_pO_n$ and $CH_iO_m^-$ represents the general formula of biomass and organic carbon, respectively. From the equation one can conclude that growth is followed by pH decrease. Furthermore, apart from the availability of the carbon source, molecular oxygen can also become a growth limiting factor. Hence sufficient aeration is a key factor for heterotrophic growth (Perez-Garcia et al. 2011). Dark respiration rates in autotrophic cultures fluctuate with cell cycle as they are coupled with growth reaching 20-30% of the growth rate in optimal conditions (Perez-Garcia et al. 2011).

The attractiveness of heterotrophic microalgae cultivation is its similarity to well-established microbial fermentation processes with equipment and operating conditions already thoroughly studied and optimized enabling high density cultures, easing and lowering the costs of harvesting (Perez-Garcia et al. 2011). The simplicity of operation translates into a reliable and reproducible growth process that culminates in a product of

consistent quality vital to industrial operations. On the other hand, substrate cost should not be underestimated as its incorporation into biomass is not fully efficient. The energy-carbon ratio of glucose and acetic acid is 114 kcal mol of C^{-1} and 105 kcal mol of C^{-1}, respectively, which is not enough for the total conversion of substrate to biomass as the energy-carbon ratio of microalgae biomass is 138 kcal mol of C^{-1} (Lee et al. 1996). In this way there is a stream of carbon that is lost from the system in the form of CO_2. Moreover, along with the restricted number of microalgae species capable of heterotrophic growth, this method makes it impossible to obtain photosynthetic products as their production is light inducible (Perez-Garcia et al. 2011).

2.2.3. Mixotrophic growth

Mixotrophy is commonly defined as a mixture of photoautotrophy and heterotrophy and it is attributed to microalgae that in some way have a hindered capability of assimilation of CO_2 requiring an extra supply of organic carbon to grow in the light (Neilson & Lewin 1974). Energy for maintenance and biosynthesis is obtained by catabolizing organic compounds and converting light energy to chemical energy via photosynthesis (5) (Hata et al. 2000; Chojnacka & Marquez-Rocha 2004).

$$dCH_iO_m^- + bCH_4N_2O \xrightarrow[kcal_{hv}]{kcal_{CH_iO_m}} C_qNH_pO_n + + \zeta H^+ + \beta OH^- \qquad (5)$$

In this scenario external CO_2 is consumed in very small amounts as CO_2 released by respiration can be entrapped and reused through photosynthetic pathways under sufficient light intensities (Neilson & Lewin 1974; Chen et al. 2011; Lee et al. 1996; Hata et al. 2000; Chojnacka & Marquez-Rocha 2004). In the same way O_2 originated from photosynthesis is consumed in the catabolism of organic substrates (Chojnacka & Marquez-Rocha 2004). Ideally the rate of photosynthesis would then equalize the respiration rate generating a self-balanced production-consumption scenario. However, this is not verified in real systems due to substrate limitations (Chojnacka & Marquez-Rocha 2004) since despite being available inside the cell compartment, CO_2 and O_2 must be transported in and out of the chloroplast and the mitochondria according to their metabolic needs. The influence of light and organic carbon, considered growth enhancing substrates, can be described by additive growth kinetics as the cell uses simultaneously the two energy sources (Chojnacka & Marquez-Rocha 2004):

$$\mu = \left(\mu_{max,I}\frac{I}{I+K_I}\right) + \left(\mu_{max,S}\frac{S}{S+K_S}\right) \quad (6)$$

In the exponential phase light energy appears to be the main source of ATP and as the culture ages and cell concentration increases, nearly all ATP produced comes from the organic substrate due to the shadow effect caused by high cell density (Yang et al. 2000). Therefore improvement in biomass productivity is possible through mixotrophic cultivation even under low light intensities or when the culture is in the linear growth phase (Chojnacka & Marquez-Rocha 2004; Ogawa & Aiba 1981). Additionally, the maximum specific growth rate of *Chlorella vulgaris* in mixotrophic culture is significantly higher than in heterotrophic ones (Lee 2001; Ogawa & Aiba 1981; Heredia-Arroyo et al. 2011). In fact, the maximum growth rate of a mixotrophic culture equals the sum of the maximum growth rate of a photoautotrophic and a heterotrophic culture. Furthermore, addition of DCMU – 1-(3,4-dichlorophenyl)-3,3-dimethylurea (a potent photosynthesis inhibitor) results in a decrease of mixotrophic growth rate to the heterotrophic level (Ogawa & Aiba 1981). This could be due to the fact that while in heterotrophy the surplus carbon molecules are loss as carbon dioxide, in mixotrophy the same excess can be rechanneled to biomass through photosynthesis using light energy.

Mixotrophy is thereby a more stable and reliable metabolic state for the industrial cultivation of microalgae biomass as the cells will be less dependent on photosynthesis and, consequently, on local weather conditions while expressing photosynthetic pigments. In this way, despite avoiding the problem of light limitation affecting photoautotrophic cultures, mixotrophic growth, by the use of organic substrates and impossibility of reactor sterilization is more susceptible to bacterial contamination (Chen et al. 2011). The continuous small quantity addition of the organic substrate as opposed to less frequent high quantity additions is the proposed solution to favour higher microalgal biomass productivity and prevent bacterial proliferation (Perez-Garcia et al. 2011).

2.2.4. Photoheterotrophic growth

Some species are also able of incorporating organic molecules even when they cannot grow in the absence of light. On these conditions, when the organic substrate is the sole or main source of carbon for cell growth, the metabolism is designated as photoheterotrophy (Neilson & Lewin 1974; Chojnacka & Marquez-Rocha 2004). These

microalgae are unable to incorporate organic substrates in low light intensities or by addition of DCMU while heterotrophs have this ability (Neilson & Lewin 1974; Chojnacka & Marquez-Rocha 2004). Therefore, photoheterotrophic cultivation requires light as the energy source and organic carbon as the carbon source at the same time resulting in the multiplicative kinetics described below (7).

$$\mu = \left(\mu_{max,I}\frac{I}{I+K_I}\right) \times \left(\mu_{max,S}\frac{S}{S+K_S}\right) \quad (7)$$

By this equation both substrates are defined as essential to growth since in photoheterotrophy, they are the sole source of energy and carbon, respectively. The proposed metabolic mechanism assumes that energy in the form of ATP/NADPH is obtained from light and used in the photolysis of organic carbon for biomass production resulting in the production of oxygen (Chojnacka & Marquez-Rocha 2004). In this way, as opposed to mixotrophy where CO_2 is a product of the oxidative catabolism of organic carbon, photoheterotrophy does not produce carbon dioxide. Additionally, in this trophic state oxygen comes from photolysis rather than from photosynthesis (8) (Chojnacka & Marquez-Rocha 2004).

$$CH_iO_m^- + bCH_4N_2O \xrightarrow{kcal_{hv}} C_qNH_pO_n + \varepsilon H_2O + \Phi HCO_3^- + \zeta H^+ + \alpha O_2 \quad (8)$$

2.2.5. The supply and metabolism of nutrients in mixotrophic growth conditions

Carbon is the main element in microalgal biomass composing about 50% DW of the cell (Markou et al. 2014). This value further increases when cells experience nutrient limiting conditions. Common to all trophic states is the variation of the efficiency of assimilation of inorganic or organic carbon into biomass with strain, light intensity and growth phase (Chojnacka & Marquez-Rocha 2004). In non-photoautotrophic conditions, microalgae can assimilate a variety of organic carbon substrates: glucose, acetate, glycerol, fructose, sucrose, lactose, galactose (Chen et al. 2011; Perez-Garcia et al. 2011; Neilson & Lewin 1974; Yeh et al. 2012; Chojnacka & Marquez-Rocha 2004; Markou et al. 2014; Ogawa & Aiba 1981). These can be supplemented in their pure form or in inexpensive industrial surplus streams in order to improve the cost efficiency of the operation as the cost of organic carbon can be significant (Chen et al. 2011; Perez-Garcia et al. 2011).

Nitrogen, apart from hydrogen and oxygen, is the second most abundant constituent of microalgal biomass composing up to 14% DW as it incorporates into nucleic acids, amino acids and pigments (Markou et al. 2014). Moreover nitrogen plays an important role in growth and metabolism regulation making it a crucial element in the intensive cultivation of microalgae (Choochote et al. 2012). It can be provided in inorganic forms such as NO_3^-, NO_2^-, NO or NH_4^+ or through organic molecules such as urea, peptone, yeast extract, amino acids and purines (Markou et al. 2014; Perez-Garcia et al. 2011; Choochote et al. 2012).

Carbon and nitrogen metabolism are strongly related as they share the carbon skeletons devised from respiration and the energy generated in the tricarboxylic acid (TCA) cycle (Perez-Garcia et al. 2011).

2.2.5.1. Carbon source – acetate and carbon dioxide

Acetate is very commonly used as carbon source in microbial fermentation processes. *Chlorella* spp. cells assimilate acetate into the cytoplasm by a monocarboxylic/proton co-transporter protein (Perez-Garcia et al. 2011). Inside the cell acetate is coupled with coenzyme-A at the expense of one ATP molecule originating acetyl-CoA (**Figure 2.2.5.1.1**) (Perez-Garcia et al. 2011; Ingraham 2006).

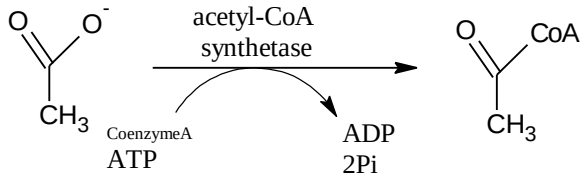

Figure 2.2.5.1.1 – Acetate incorporation into acetyl-CoA by acetyl-CoA synthetase.

Acetyl-CoA is a metabolic intermediate common to a wide variety of pathways. Particularly for the acetate metabolism of *Chlorella* spp., acetyl-CoA can be incorporated by two main pathways depending on the culture conditions (Figure 2.2.5.1.2). The TCA cycle that takes place in the mitochondrion can result in (1) net production of chemical energy (ATP) and reductive power (NADH) releasing the carbon from acetate as CO_2, or in (2) protein precursors (α-ketoglutarate), via glutamine synthase/ glutamine oxoglutarate aminotransferase (GS/GOGAT) cycle, resulting in the incorporation of one acetate carbon into proteins, the other being released as CO_2. On the other hand, the glyoxylate shunt taking place in the glyoxysome bypasses the CO_2-evolving steps of the TCA cycle producing C_4-skeletons for (1) biosynthesis or (2) to

restore intermediates of the carbon metabolism (succinate, malate, fumarate), resulting in net incorporation of carbon. These two pathways share most of the intermediate molecules and studies suggest that ultimately acetate can be incorporated (1) into intermediates of the TCA cycle (malate, succinate and citrate) and to amino acids commonly associated to it (aspartate and glutamate) (Syrett et al. 1964; Merrett & Goulding 1968; Goulding & Merrett 1966) if the culture conditions are favourable to high growth rates, or (2) into saturated, mono- and di-unsaturated fatty acids (Neilson & Lewin 1974; Liang et al. 2009), high energy reserve molecules, if growth is somewhat constrained. Additionally, microalgal cells can also mobilize acetyl-CoA for the carbohydrate metabolism by gluconeogenesis (Johnson & Alric 2013). The efficiency of acetate incorporation, in the most favourable scenario, is very high as there are 1.6 carbon atoms being assimilated out of the two available per molecule of acetate (Fujita 1959). By isotope experiments it was found that all the methyl carbon in acetate is converted into cell material while two-thirds of the carboxyl carbon are incorporated into biomass and the remaining is discharged as CO_2 (Fujita 1959).

The amino acid biosynthesis via GOGAT cycle is the first and most important common branch of nitrogen and carbon metabolisms (Guerra et al. 2013). The carbon skeletons necessary for amino-acid synthesis are supplied via TCA cycle mainly by α-ketoglutarate (Figure 2.2.5.1.2).

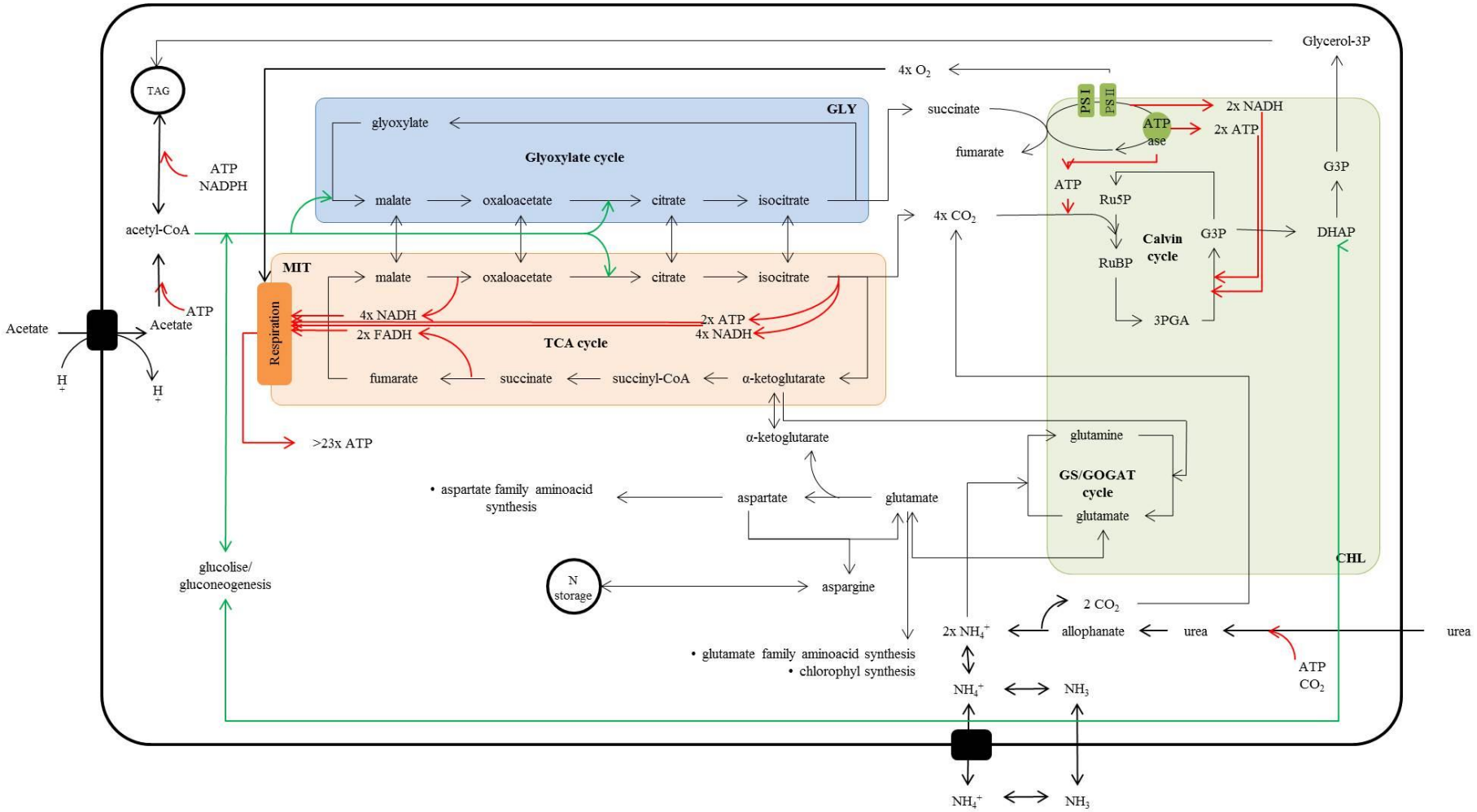

Figure 2.2.5.1.2 – Metabolism of acetate and urea in mixotrophic cells of *Chlorella vulgaris*. TAG – triacylglycerol; G3P – glyceraldehyde-3-phosphate; DHAP - Dihydroxyacetone phosphate; PS I –photosystem I; PS II – photosystem II; Ru5P – ribose-5-phosphate; RuBP - Ribulose-1,5-bisphosphate; PGA – phosphoglyceric acid; CHL – chloroplast; GLY – glyxosome; – cytoplasm; MIT – mitochondria. (Based on Guerra et al. 2013; Johnson & Alric 2013)

The regulation of the acetate metabolism is complex. In dark heterotrophic conditions in the presence of acetate, *Chlorella* spp. cells induce the expression of isocitrate liase and malate synthetase, the enzymes responsible for the glyoxylate cycle (Perez-Garcia et al. 2011; Neilson & Lewin 1974; Merrett & Goulding 1968). On the other hand, despite being operational, the activity of the TCA cycle is not enhanced (Perez-Garcia et al. 2011; Neilson & Lewin 1974). Short-term kinetic studies using 2-^{14}C acetate reveal the rapid formation of citrate and malate indicating the simultaneous operation of the glyoxylate and TCA cycles (Neilson & Lewin 1974). However, in the light *Chlorella* spp. incorporates much more acetate than when it is grown in the dark (Goulding & Merrett 1966). In these conditions acetate is incorporated into polysaccharides, nucleic acids and amino acids of both glutamate and aspartate families (Syrett et al. 1964; Neilson & Lewin 1974). In the presence of light, inorganic CO_2 has a significant effect on the assimilation of acetate. When CO_2 is present, the synthesis of the glyoxylate cycle enzymes is repressed – only 6% of isocitrate liase expression levels found in dark conditions (Perez-Garcia et al. 2011; Neilson & Lewin 1974; Merrett & Goulding 1968; Fujita 1959) while in its absence isocitrate liase is synthetized up to 71% (Neilson & Lewin 1974). Radioactive labelling of acetate shows a difference in the primary products of acetate assimilation as its incorporation is observed in succinate (65%) and glycollate (15%) within 10 s time (Neilson & Lewin 1974; Merrett & Goulding 1968; Goulding & Merrett 1966). It is known that glycollate is a net product of photorespiration and that the photoassimilation of acetate is dependent on the oxygen-evolving system of photosynthesis in *Chlorella* sp. (Neilson & Lewin 1974; Goulding & Merrett 1966). In this way, despite scientific evidence being scarce on light acetate metabolization in *Chlorella* spp., one can postulate that succinate can be transformed into fumarate in a photorespiration process that would result in the production of glycollate leading to the results observed by (Goulding & Merrett 1966). Similar conclusions are reported for *Chlamydomonas reinhardtii* which is known to support acetate uptake by cyclic photophosphorylation (cyclic electron transfer in photosystem I) (Johnson & Alric 2013). This is also concurrent with the requirement of both photosynthesis and glucose assimilation for support of maximum growth rate in *Chlorella vulgaris* UAM 101 (Martinez & Orus 1991). Nevertheless, glycollate is not excreted or accumulated by acetate-grown *Chlorella* spp. as it happens when glucose is

the carbon source. On the contrary, it can be transformed into glyoxylate, glycine, serine, hydroxypiruvate and glycerate (Neilson & Lewin 1974).

In the same way as the presence of light stimulates organic carbon assimilation, acetate seems to induce higher photosynthetic rates as well (Martinez & Orus 1991). The net rate of O_2 evolution is the algebraic sum of its photosynthetic generation and respiratory consumption. Once the respiratory rate largely increases with organic carbon assimilation and the net rate of O_2 evolution remains the same, there must be an increase in the photosynthetic rate as well (Martinez & Orus 1991). The assimilation of external inorganic carbon (C_{ino}) when cells are in the presence of an organic source is also of importance. It was demonstrated that cells grown aerobically on glucose with no supplementation of CO_2 exhibited the same photosynthetic affinity for C_{ino} as cells adapted to high concentrations of CO_2. This might be due to the fact that carbon resulting from respiration of the organic substrate is an alternative source for C_{ino} fixation (Martinez & Orus 1991). In fact, the assimilation of external C_{ino} is somewhat inhibited in cultures adapted to organic carbon despite the higher photosynthetic rates exhibited. These rates were shown to be higher than that of cells adapted to low CO_2 concentrations, i.e., of super-efficient C_{ino} fixating cells (Martinez & Orus 1991). Accordingly, Neilson & Lewin 1974 also reported the repression of C_{ino} fixation when *Chlorella* sp. cultures were grown on acetate.

2.2.5.2. Nitrogen source – urea and ammonia

Microalgae usually take up the most readily accessible form of nitrogen available in the medium. This is based on a combination of factors such as the oxidation level of the N atom in the molecule or its easiness of assimilation by the cell. For instance, despite having the same oxidation state that urea, ammonium is passively diffused into the cytoplasm while urea has to be actively transported (Molloy & Syrett 1988).

Urea is a cheap and effective organic nitrogen source for cultivation of *Chlorella* sp. cells as it can provide an extra source of reduced carbon (Choochote et al. 2012; Hodson & Thompson 1969; Markou et al. 2014; Hattori 1960). Urea is passively diffused and/or actively transported into the cells. It can be hydrolysed into ammonium and bicarbonate in a single step by urease or in a two-step carboxylation-hydrolysis by urea carboxylase/hydrolase (Perez-Garcia et al. 2011; Hodson & Thompson 1969; Markou et al. 2014; Molloy & Syrett 1988). The former, most metabolically expensive route, is the

one used by *Chlorella vulgaris* as this species does not possess urease (Figure 2.2.5.2.1) (Hodson & Thompson 1969; Perez-Garcia et al. 2011; Caspi 2007).

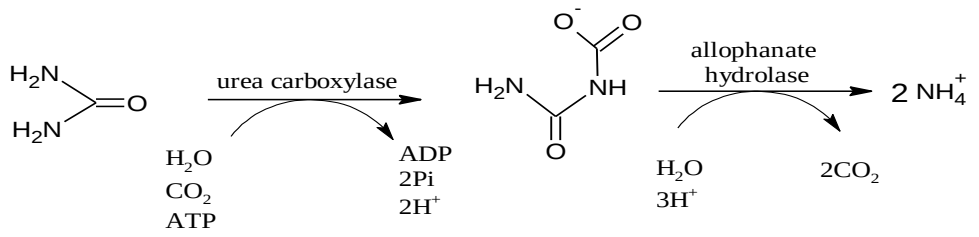

Figure 2.2.5.2.1– Urea degradation process by *Chlorella vulgaris.*

While the CO_2 produced by urea degradation is readily incorporated into the alcohol-soluble fraction of the biomass in aerated conditions, when the culture is flushed with 10% inorganic CO_2, its assimilation is eliminated (Hodson & Thompson 1969; Hattori 1960). Similarly to acetate, urea metabolism is also influenced by light. When in dark conditions, almost every carbon atom of urea was released to the medium as carbon dioxide as opposed to being incorporated to cell material in the presence of light in proportions up to 63% (Hattori 1960).

The resultant ammonium molecules can then be incorporated by the cells into amino acids by the usual pathways or be stored in vacuoles (Hodson & Thompson 1969; Perez-Garcia et al. 2011). In auto or heterotrophic conditions ammonium can be catalysed (1) by glutamine and glutamate synthases generating two molecules of glutamate and one of α-ketoglutarate or (2) by glutamate dehydrogenase that yields one molecule of glutamate by the reductive amination of α-ketoglutarate (Perez-Garcia et al. 2011). While the first alternative is the primary pathway for nitrogen assimilation, the second is viewed as a shunt to ensure mitochondrial function is not affected and to remobilize nitrogen compounds within the cell and its contribution is not significant in glutamate formation (Perez-Garcia et al. 2011). Following, aspartate can be obtained by transamination with oxaloacetate by aspartate aminotransferase and used along with glutamine by asparagine synthetase to yield one molecule of asparagine and other of glutamate. In this way, all the building blocks for amino acids, nucleotides, chlorophylls, polyamines and alkaloids can be obtained from glutamine, glutamate, aspartate and asparagine (Perez-Garcia et al. 2011). Increasing urea concentration has a positive effect on growth for concentrations up to 800 mg L^{-1} (Choochote et al. 2012). However at high metabolic rates, urea degradation can produce excess of ammonium that can be actively transported to the medium. At culture pH higher than pK_{NH_3} (9.25)

the predominant form of buffer system is ammonia that being able to diffuse passively through the cell wall hinders the control of intracellular concentrations which could become toxic (Markou et al. 2014). Free ammonia at low concentrations (2 mM) induces photo-damage onto photosystem II of microalgae cells affecting its viability (Markou et al. 2014). The effect of simultaneous presence of more than one nitrogen source in the medium is not fully understood. Total nitrogen uptake rate is slightly higher when both urea and ammonium are available in the culture medium than when a single source is present. However this rate remains lower than their total (Healey 1977). On the other hand, ammonium is known to repress urea uptake and vice-versa although they do not appear to directly compete. In fact it is postulated that a common internal rate limiting step may regulate the uptake of both N-sources (Healey 1977). Some authors agree that the presence of ammonium/ammonia in the medium can repress urea assimilation as less energy is necessary for its uptake (Markou et al. 2014; Perez-Garcia et al. 2011). The concentration at which the repression occurs is species dependent. For *Chlorella emersonii*, ammonium concentrations of 1 mM are enough for urea uptake to be completely repressed (Molloy & Syrett 1988). Also, the metabolic state of the cells has influence in this mechanism. As nitrogen assimilation requires carbon skeletons for keto-acids synthesis, in N-depleted cells with higher carbon reserves nitrogen uptake is more efficient (Healey 1977; Guerra et al. 2013) and urea uptake is more affected by the presence of ammonium (Molloy & Syrett 1988).

Chapter 3

3. Overview of the Algafarm biomass production process

Algafarm, a joint project by Secil/CMP and A4F aimed at microalgal biomass production for food and feed markets is located at Pataias, in Portugal (temperate climate zone). The current project took place in the spring/summer period of 2014. The presented data were obtained between April 7th and June 18th.

3.1. Culture scale-up

Chlorella vulgaris seed inoculum is grown photoautotrophically in a controlled environment. The cultures grow at 20 ºC with a light:dark cycle of 24:0 in 5 L vessels with constant aeration and periodical 4 s injections of pure CO_2. The nitrogen source is nitrate. Following, the culture is scaled-up in a proportion of 1:20 to a 1 m^3 Green-Wall flat panel photobioreactor housed in a greenhouse, *i.e.*, a semi-controlled environment. Here cells are subjected to a circadian light:dark cycle. Finally, the culture is transferred to an outdoor 10 m^3 photobioreactor (PBR_S) as the one described in **Figure 3.2.1**. At this fed-batch stage the trophic state is altered to mixotrophy by feeding with an acetate-urea solution. When sufficiently concentrated the culture is scaled-up to a 37 m^3 photobioreactor (PBR_M) in fed-batch and later to a 100 m^3 production photobioreactor (PBR_L). From here forward the culture is periodically harvested (semi-continuous mode maintaining an average dry weight of 1 g L^{-1}.

3.2. Photobioreactor design and operation

In the present study, a culture was followed from PBR_S to PBR_L. All photobioreactors are located outdoors implying a circadian light:dark cycle and day/night temperature fluctuations. During operation time, cultures were kept in homogeneous suspension throughout the reactor. Microalgal culture flow (represented in **Figure 3.2.1**) comprised a dark phase inside the tank where basal and feeding media or other occasional supplements could be added as well as an illuminated phase where cells run through transparent tubes. Both the tank (dark phase) and the transparent tubes (light phase) increase in size although volumetric proportions are maintained. Some relevant operational conditions were monitored online and automatically controlled: (1)

turbidity, (2) temperature which was kept lower than 28 ºC by water sprinkling over the PBR and (3) pH that was maintained under 7.8 by injection with pure carbon dioxide and also regulated by the addition of acidic culture medium. The pH values overtime can be observed in Figure 4.3.3.1.

The basal medium consists on a balanced mixture of key macro and micronutrients: P, K, Mg, Co, Cu, Fe, Mn, Mo and Zn. On the other hand, carbon and nitrogen are fed through an acetate and urea solution with an overall 7.5:1 C:N ratio – feeding medium. On occasion, urea can be supplemented in order to maintain the intended concentration in the culture medium. While the former is provided every day during the photoperiod and according to biomass growth, basal medium is only added upon inoculation or partial harvesting of the cultures. This operation always takes place at night wherein the amount of biomass harvested is variable according to its concentration and the production needs.

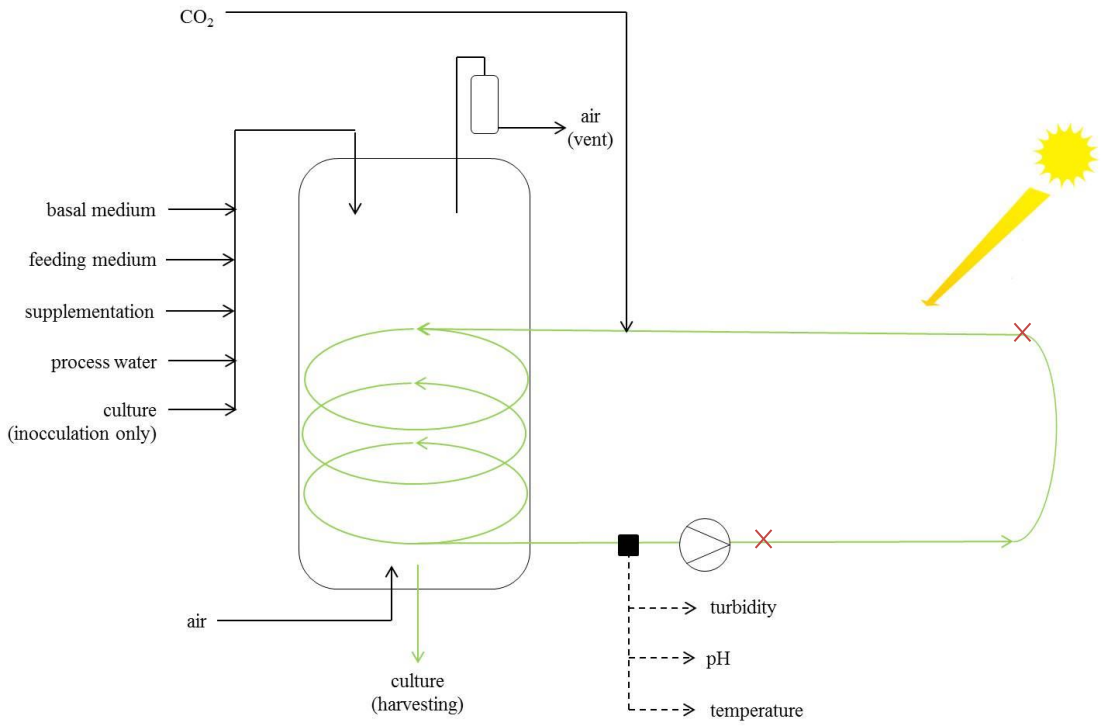

Figure 3.2.1– Simplified photobioreactor design diagram. Green arrows illustrate culture flow, black arrows indicate inputs or outputs (measurements in dashed lines) and the red crosses the possible sampling points.

Sampling points are illustrated in Figure 3.2.1. In order to determine day to day monitoring of nutrient consumption and culture grow samples were taken before and after the feeding periods, at early morning (~9 h) and late afternoon (~19 h), respectively.

The control of the organic carbon feed to microalgal cultures is of crucial importance. In one hand acetate concentration must be kept low so that proliferation of contaminant species, such as bacteria, do not proliferate (Lee 2001). On the other, it is important that this low concentration is not growth limiting to the microalgal cells so a critical balance should be achieved. Hence, feeding rate is adjusted in proportion to microalgal biomass growth: upon constant acetate feeding, the higher the growth rate, the higher the pH increase (Figure 4.3.3.1). Thus, in order to maintain the pH of the culture approximately constant throughout microalgal growth, feeding was increased or decreased accordingly. As previously discussed in Chapter 2, mixotrophic growth is also related to light intensity. Therefore, in order to partially automate the feeding rhythm, a timer was set (and adjusted when necessary) to distribute feeding periods, at a fixed feeding rate, in relation to light intensity. These higher feeding streams coupled to higher incident radiation are observable in . A typical day situation is presented in Figure 3.2.2.

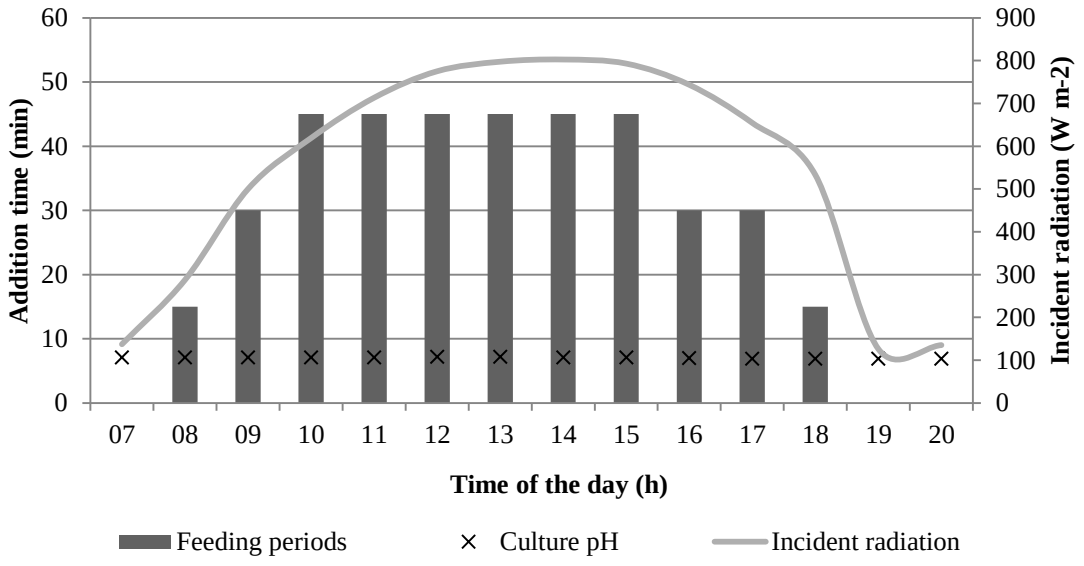

Figure 3.2.2 – Graphic representation of feeding periods and its relation to incident radiation and culture pH. Data from day 11-06-2014.

Chapter 4

4. The supply and metabolism of nutrients in industrial mixotrophic cultures

4.1. Introduction

Large-scale photoautotrophic cultivation of microalgae is slow, highly dependent on weather conditions and concentrated cultures are very difficult to obtain (Liang et al. 2009). Mixotrophic cultivations enable high density cultures that maintain production of photosynthetic pigments at the same time that reduces weather liability (Pagnanelli et al. 2014). Despite being implemented in Asian countries since 1964, mixotrophic cultivation of microalgae has not been fully described (Richmond 2007). Namely, the relationship between the assimilation of organic carbon and light, the incorporation of CO_2 derived from the respiration of such organic sources and the effect of the presence of inorganic carbon dioxide.

Some studies suggest that mixotrophic growth is the algebraic sum of heterotrophic and autotrophic metabolisms functioning independently and noncompetitively (Richmond 2007; Lee et al. 1996). As follows, the increase in mixotrophic growth resulting from light incidence is equivalent to the photoautotrophic growth in the same conditions (Richmond 2007). *Chlorella vulgaris* can grow on a vast number of organic carbon sources. Acetate is a cheap alternative and has the same theoretical yield of incorporation as glucose (0.8 $g_X g_{glucose}^{-1}$) (Lee et al. 1996; Yeh et al. 2012). Furthermore, its addition to the medium can be regulated by pH as it decreases with addition and increases with consumption (Richmond 2007; Yeh et al. 2012). Urea is therefore a favourable nitrogen source in such a system as its addition does not affect the pH (Richmond 2007; Scherholz & Curtis 2013; Lee et al. 1996).

As industrial conditions often differ from the conditions in the aforementioned studies, this chapter aims to analyse biomass growth and nutrient consumption of a large scale mixotrophic *C. vulgaris* culture during the course of scale-up. Moreover, the suitability of the feed composition in C and N and of acetate and urea as carbon and nitrogen sources was evaluated.

4.2. Materials and methods

4.2.1. Quantification of biomass growth

Samples were collected daily at 9h00 and 19h00. For consistency with weekends and holidays, growth was evaluated using turbidity *versus* dry weight calibration curve (appendix A.1.1). To establish the correlation, online turbidity values of the cultures are obtained by a Hach-Lange sensor installed in the PBR as described in **Figure 3.2.1**. On the other hand, culture samples were filtered using pre-weighted Whatman GF/C filters and dried until constant mass is obtained using a Kern DBS 60-30 electronic moisture analyser.

4.2.2. Quantification of urea concentration

Samples were collected daily at 9h00 and 19h00 and centrifuged using a Hermle centrifuge. When immediate analysis was not possible the supernatant was stored at -20 ºC. Urea concentration was determined by a colorimetric method similar to the one described by Watt & Chrisp 1954. The colorimetric agent, an acidic ethanoic solution of p-dimethylaminobenzaldehyde (0.003 mM), was added to the samples (proportion of 0.5:2 v/v) and the absorbance of the mixture was determined 10 min later at 418 nm using a Thermo Scientific™ Genesys 10S UV-Vis spectrophotometer. The calibration curve and associated error are presented in appendix A.1.2.

4.2.3. Quantification of ammonium concentration

Samples were collected daily at 9h00 and 19h00 and centrifuged using a Hermle centrifuge. The pellet was discarded and the supernatant was stored at -20 ºC for later analysis. Ammonium concentration was determined using the Sera Ammonium-Ammonia Test that by a back titration with sodium hydroxide produces a coloured solution, the absorbance at 697 nm of which is proportional to the ammonium concentration. The absorbance was determined using a Thermo Scientific™ Genesys 10S UV-Vis spectrophotometer. The calibration curve and associated error are presented in appendix A.1.3

4.2.4. Quantification of acetate concentration

Samples were collected daily at 9h00 and 19h00 and centrifuged using a Hermle centrifuge. The pellet was discharged and the supernatant was stored for later analysis at -20 ºC. Acetate concentration was determined by the Megazyme K-ACETRM 07/12

method (Appendix A.1.4) by which a sequence of specific enzymatic reactions produces NAD^+. As stated by the manufacturer, the amount produced is stoichiometrically equal to the amount of acetate in the sample. The consumption of NADH for NAD^+ formation is then measured by the decrease in absorbance at 340 nm. The measurements were performed using a Thermo Scientific™ Genesys 10S UV-Vis spectrophotometer.

4.2.5. Estimation of biomass elemental composition

The elemental composition of the biomass produced was estimated using a report of the macronutrient analysis of four Algafarm batches by Silliker, Inc (Appendix A.2). To estimate the elemental composition of the cells, carbohydrate and dietary fibre content was assumed to be 100% glucose and cellulose, respectively. Fat and protein content were segmented based on the distribution of fatty and amino acids in the full biochemical analysis performed to batch LA by Silliker, Inc.

4.2.6. Monitoring of the microorganism population

The contamination load and cellular state of the *Chlorella vulgaris* cultures followed in this study were routinely monitored by the Algafarm production team.

4.3. Results and discussion

4.3.1. Biomass growth

C. vulgaris growth curve is presented in Figure 4.3.1.1. Cumulative growth was considered in order to eliminate the dilution effects of the scale-up and partial harvesting processes. Hence total volume of the culture was considered (9). The relative error includes the turbidity *versus* dry weight correlation error as well as the error of volume estimation.

$$V_{total} = V_{PBR\_S} + V_{PBR\_M} + V_{PBR\_L} + \sum V_{partial\ harvesting} \quad (9)$$

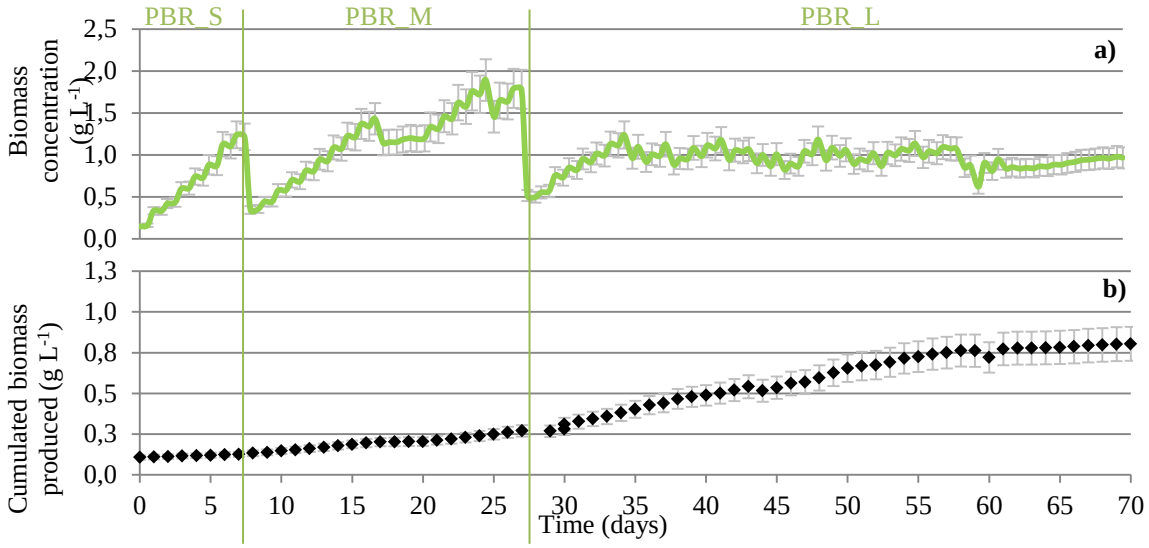

Figure 4.3.1.1– Graphic representation of *C. vulgaris* biomass concentration along the cultivation period (a) and cumulated growth curve (b).

C. vulgaris adaptation to the organic substrate is very fast. This is noted in both the biomass concentration in Figure 4.3.1.1 as in the total carbon in where only in day 1 acetate concentration in the medium is higher than 1 mM. The growth rate is maximum in PBR_L and only after the 60th day does the culture enter the stationary phase. The abrupt downward variations observed on days 8, 17, 25 and 28 relate to the dilution of the culture due either to the inoculation of a bioreactor or to the partial harvesting of the culture. In the same way, the biomass concentration is maintained approximately constant from day 35 onward given the periodically partial harvesting that the culture is subjected to. Additionally, the plateau observed on the biomass concentration curve (Figure 4.3.1.1a)) between the 18th and 20th days is the result of an erroneous suppression of the feed stream [1].

Table 4.3.1.1 – Growth parameters of *C. vulgaris* mixotrophic culture throughout scale-up.

		PBR_S	PBR_M	PBR_L	Global
Maximum growth rate, μ_{max}	d^{-1}	0.588	0.457	0.673	0.673
Biomass productivity, P_X	$g\ L^{-1}\ d^{-1}$	0.139	0.112	0.066	0.087

The same is observed by the parameters described in Table 4.3.1.1. The maximum growth rate corresponds to the maximum value of the growth rates recorded in the considered time period. Maximum growth rate is obtained when the culture is in PBR_L. Here, the routine harvesting keeps the culture more diluted (approximately at 1

[1] This phenomenon is analysed in detail in Section 4.3.3.

g L^{-1}, Figure 4.3.1.1) resulting in easier light penetration. Biomass growth in mixotrophic conditions has been demonstrated to increase with light in accordance with the data obtained in previous studies (Yang et al. 2000; Chojnacka & Marquez-Rocha 2004). Despite being below average considering data in Table 2.2.1 for mixotrophic growth of *Chlorella* spp., the maximum growth rate obtained in this study is satisfactory considering the scale of the process and especially the lack of sterile conditions. Biomass productivity was calculated given the total variation in biomass relative with the considered time period (PBR_S, PBR_M, PBR_L or total cultivation time). Productivity declines along the scale-up. The increase in system volume is associated with a decrease in turbulence and culture flow velocity which can result in higher biofilm formation and inefficient substrate distribution throughout the reactor that can justify the decrease in biomass productivity of PBR_M and PBR_L.

4.3.2. **Nutrient consumption**

Nutrient consumption can be analysed by the data in and Table 4.3.2.1. Substrate yields where calculated considering the total amount of substrate fed to the system as well as the total biomass produced in the time period considered (PBR_S, PBR_M, PBR_L or total cultivation time).

Table 4.3.2.1 – Substrate yields and C:N ratio consumed by *C. vulgaris* mixotrophic cultures throughout scale-up. [2]

		PBR_S	PBR_M	PBR_L	Global
Acetate yield, $Y_{X/HAc}$	$g_X\, g_{HAc}^{-1}$	0.78	0.71	0.65	0.67
Urea yield, $Y_{X/Ur}$	$g_X\, g_{urea}^{-1}$	3.17	3.72	3.43	3.61
Carbon yield, $Y_{X/C}$	$mol_{C\text{-}X} mol_C^{-1}$	0.77	0.76	0.75	0.76
Nitrogen yield, $Y_{X/N}$	$mol_{N\text{-}X} mol_N^{-1}$	0.56	0.74	0.69	0.72
Feed yield, $Y_{X/S}$	$g_X\, g_S^{-1}$	0.44	0.40	0.39	0.39
C:N feed	$mol_C\, mol_N^{-1}$	4.14	5.65	5.36	5.54
C:N consumed	$mol_C\, mol_N^{-1}$	10.4	7.02	5.90	7.46

Carbon in the feeding medium takes into account the molecules present in both acetate (C2) and urea (C1). From Figure 4.3.2.1 one can observe that acetate does not

[2] Note that the C:N ratio as well as the carbon and nitrogen yields are calculated according to the assumptions made in Chapter 5.

accumulate once for different feed values its concentration in the medium remains approximately zero. However, yields of acetate and carbon decrease with the increase in culture volume. The distribution of acetate throughout the length of the reactor has not been fully studied. Although occasional samples were analysed both prior and in the middle of the photosynthetic zone, acetate concentration was not significantly different and remained close to zero. Therefore, as the feeding point is located at the top of the tank (Figure 3.2.1) and PBR scale-up implies longer transparent tubes (photic region), one can postulate that cells might be exposed to acetate in the photic region during shorter periods of time with increasing reactor volume.

Urea is maintained at higher concentrations than acetate but it does not accumulate in the medium as well. Urea has to be systematically available in order to prevent N-depletion effects on the cultures. The resultant accumulation of high energy molecules would not favour the intended biomass production, since cells cease division. Additionally, absence of nitrogen would prevent chlorophyll synthesis decreasing cell ability to harvest energy from light consequently hindering the incorporation of acetate carbon and the uptake of urea (Healey 1977; Quinn et al. 2011). Such interactions can be observed in as a decrease in urea concentration results in a slight increase in the acetate levels recorded. On the other hand, as recirculation of growth medium upon partial harvesting was not implemented in this study, one cannot afford to waste substantial amounts of urea due to this operation so a suitable balance must be achieved. Lower urea and nitrogen yields were observed in PBR_S taking into account the higher feed that was supplied at this stage. Here urea concentration was adjusted approximately to 3 mM whereas in the later phases of scale up the adjustment was to 1 mM.

As previously discussed, urea metabolism results in extracellular ammonium build-up. This can be observed in where an accumulation of ammonium is clear in the PBR_S section. In this section the concentration profiles suggest that one molecule of NH_4^+ is excreted *per* molecule of urea consumed as NH_4^+ concentration appears approximately half of the concentration of urea. However, in the remaining sections this relation does not hold as curves intersect and become equivalent. Nevertheless, ammonium (N1) can be taken up and used as N-source therefore being considered for nitrogen balance along with urea (N2). Contrary to acetate and carbon, urea and nitrogen yield is not greatly affected by scale-up. This might be due to the fact that

being present at higher concentrations than acetate, complete distribution throughout the reactor is possible independently of the scale.

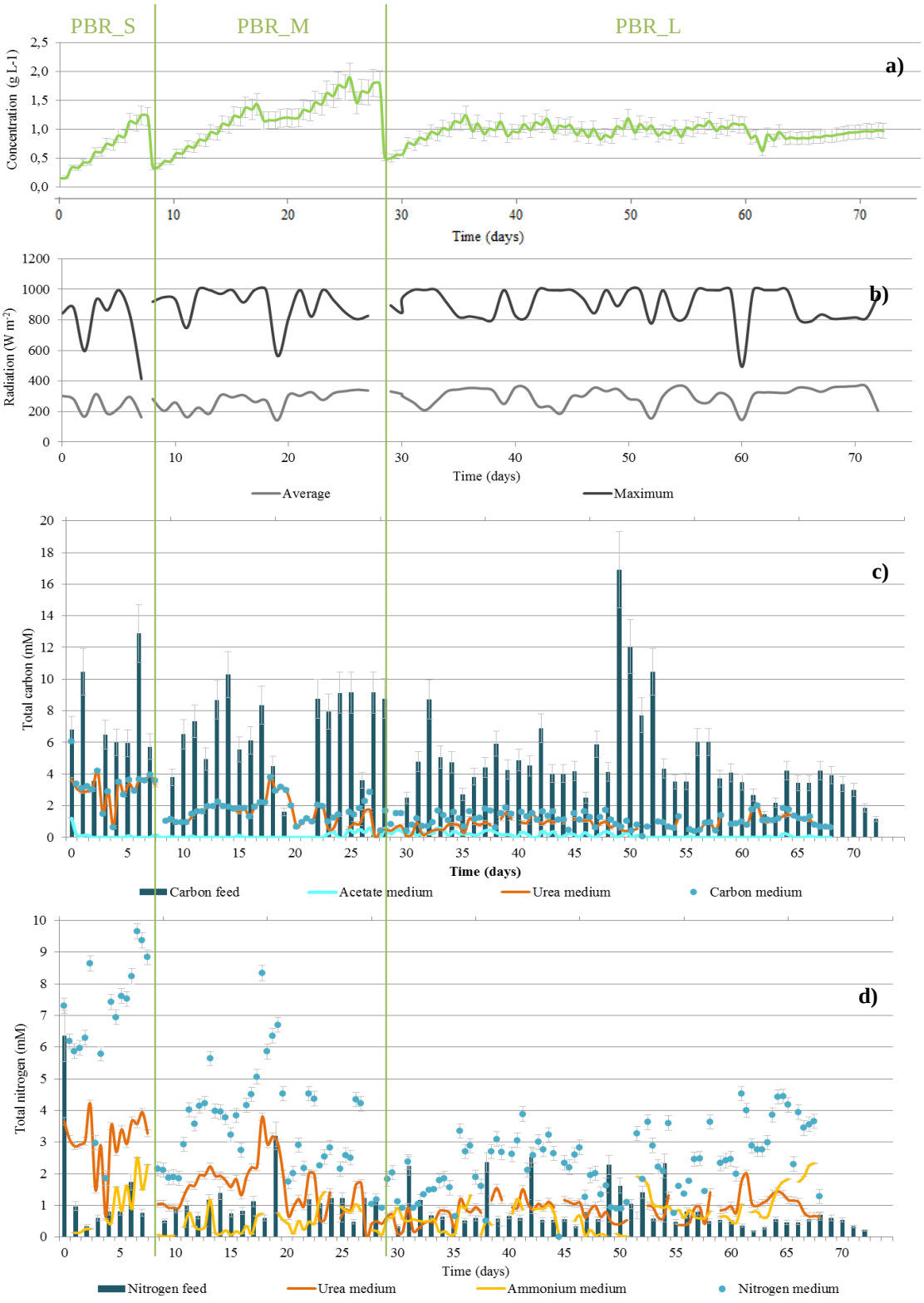

Figure 4.3.2.1 – Graphic representation of a) biomass concentration (g L^{-1}) throughout scale-up, b) maximum and average radiation (W m^{-2}), c) total carbon (mM) and d) total nitrogen (mM) in the feed and measured in the medium as opposed to the respective sources. Carbon medium represents the sum of C-Hac and C-Ur and nitrogen medium N-Ur plus N-Am.

In order to evaluate the efficiency of incorporation of the supplied carbon and nitrogen into the biomass, its elemental composition was estimated.

Table 4.3.2.2 Elemental composition of *Chlorella* spp. in different growth conditions

Trophic conditions	C	N	O	H	S	Reference
photoautotrophy	9.16	1.00	4.35	16.71	0.05	(Richmond 2007)
photoautotrophy	5.00	1.00	2.00	8.00	-	(Lin 2005)
mixotrophy	6.25	1.00	3.00	10.50	0.05	(Pollo 2012)
mixotrophy	5.84	1.00	2.85	10.39	0.001	This study

Elemental composition of *Chlorella vulgaris* has been reported to range from $C_5N_1O_2H_8$ by (Lin 2005) to $C_{9.16}N_{1.00}O_{4.35}H_{16.71}S_{0.05}$ by (Richmond 2007). The former greatly differs from the one obtained likely due to the differences in the conditions of cultivation. A similar work by Pollo 2012 resulted on a stoichiometric formula of $C_{6.25}N_{1.00}O_{3.00}H_{10.50}S_{0.05}$ for mixotrophically grown *Chlorella sorokiniana* on glucose. This formula is closer to the one estimated in this work, $C_{5.84}N_{1.00}O_{2.85}H_{10.39}S_{0.001}$, given that both cultures were grown in mixotrophic conditions.

Carbon to nitrogen ratio of the feed should be similar to the one consumed by the biomass. Correspondingly, C:N ratio consumed should be close to the one found in the biomass stoichiometric formula. This assumption is valid in PBR_L due mainly to two factors: (1) the quasi-stationary state of the culture that balances inlets (feed) and outlets (biomass harvested) and (2) to the fact that the biomass analysed for the estimation of its elemental composition originated from this type of PBR. As previously discussed, the increase in volume results in a decrease of flow turbulence coupled with culture aging, increment of the contamination load and biofilm formation. These conditions hinder biomass growth as light might not be sufficient to undergo photosynthetic respiration and consequent ATP generation. In this way, cells may shift its metabolism to higher respiration rates increasing the carbon quota that is lost as CO_2. Thus, as the incorporated carbon diminishes, the C:N ratio of the biomass might decrease in accordance with the results presented in Table 4.3.2.1.

Additionally, similarly to the work of Lee et al. 1996 one can calculate the maximum theoretical yield of acetate incorporation. Considering the biomass elemental formula estimated and that both carbon atoms of acetate are incorporated, the theoretical yield would be 0.8 g_X g_{HAc}^{-1}. This value is very close to the ones obtained

experimentally in this study. In the same conditions, for incorporation of both nitrogen atoms of urea, the theoretical yield would be 4.67 $g_X g_{urea}^{-1}$ which is higher than the values presented on Table 4.3.2.1.

4.3.3. CO_2 consumption

During cultivation, the culture suffered a major setback as the feeding medium was not sufficiently supplied from day 18 to day 20. This is easily observed by the plateau on the growth curve (Figure 4.3.1.1). Here, despite operational conditions remained controlled, the cells had to shift to a photoautotrophic metabolism. A detailed view of this situation is present below (Figure 4.3.3.1). The culture took approximately 5 to 6 h to adapt to the photosynthetic exclusive conditions and resume growth. During this stage approximately 0.03 g L^{-1} of dry weight was lost.

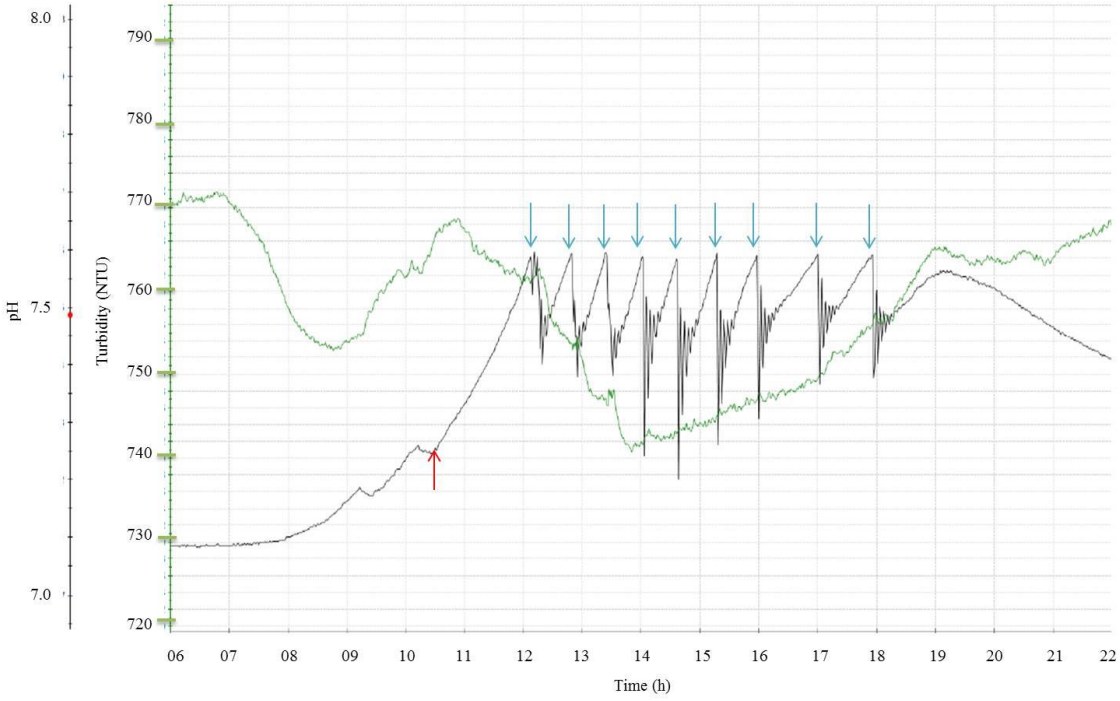

Figure 4.3.3.1– Graphic representation of the pH (presented in black on a scale of 7 to 8) and turbidity, NTU, (presented in green on a scale of 720 to 790) evolution on the 18th day of cultivation. This graphic is automatically generated from online measurements of the operation parameters. The moment the feeding was stopped is indicated by the red arrow. Blue arrows represent CO2 injections for pH control.

In addition to acetate there are a variety of inorganic carbon sources in the medium available for cell consumption. Aside from the injections for pH control the medium is permanently saturated with bicarbonate as the culture is constantly aerated. This persistent carbonation balances the system with regard to inorganic carbon. Furthermore, the time that took the cells to shift from mixotrophic growth towards

exclusive CO_2 consumption implies that they are not adapted to its consumption concomitantly with the organic substrate. One can postulate that while CO_2 from acetate respiration and urea breakdown only has to migrate between cell compartments, whereas inorganic carbon dioxide has to be imported from the culture medium by HCO_3^- transporters that might not be expressed on mixotrophic cells. This theory is supported by previously discussed observations by Neilson & Lewin 1974; Chen et al. 2011; Lee et al. 1996; Hata et al. 2000; Chojnacka & Marquez-Rocha 2004.

4.4. Conclusions

Acetate and urea were found to be effective sources of carbon and nitrogen for *C. vulgaris* growth in the conditions of the study. However, contrary to acetate, urea yield was lower than its theoretical value, so other nitrogen sources could be tested.

C:N consumption ratio decreased throughout scale-up. As the state of the culture deteriorates with cultivation time, the metabolic needs of the cells can suffer alterations that might ultimately translate to its elemental composition. A detailed biomass biochemical analysis to all scale-up stages could confirm this hypothesis thus justifying the variation in C:N consumption. If confirmed, such variations should be followed by the C:N ratio of the feed in order to better suit cellular needs. Nevertheless, the estimation of the elemental composition of the biomass was in accordance to the overall C:N ratio of consumption calculated so it can be used for the mass balance calculations. Moreover, mass balance calculations will allow further understanding of the inorganic CO_2 need.

Additionally, the decrease of the acetate yield with volume might be due to mixture problems related with scale. Therefore the possibility of adding an extra feeding points should be tested. Also, the relation between acetate assimilation and light should be accounted for when choosing the location of feeding points. To optimize mixotrophic growth, these points should be located close to or in photosynthetic zone of the reactors.

It was also very clear the complete consumption of the acetate fed to the culture. Such verification can save time and money by reducing the number of analytical procedures to be implemented. On the other hand it appears to be necessary to include the monitoring of ammonium concentration in the culture medium in order to evaluate total nitrogen levels.

Chapter 5

5. Mass balance analysis of carbon and nitrogen in industrial mixotrophic microalgae cultures

5.1. Introduction

The optimization of operational parameters for large-scale biomass production is technically challenging given the amount of process-influencing variables (Rawat et al. 2013). These challenges generally regard nutrient supply and recirculation, gas transfer and exchange, delivery of photosynthetically active radiation, culture integrity and harvesting (Christenson & Sims 2011).

Material and energy balances are of great importance to the industry. The material balance of entering and exiting streams of an operation is key to understand the importance of each single element on the larger picture. Additionally it facilitates the assessment and control of operational yields. Over recent years, this has assumed great importance given the increasing cost of energy and feedstock, as well as the need for economic and environmental sustainability. Especially for biological industries, production control is crucial as reliably consistent production is harder to achieve and raw materials are normally more expensive than the ones of traditional production units.

In batch operations, "everything that enters a process must come out". The same is true for a continuous operation if a delimited period of time is considered. The biomass production system of the study operates in semi-continuous mode so the definition of the time period for the mass balance is of importance. Trophic interrelations and many alternative metabolic pathways for mixotrophic growth of *C. vulgaris* made it difficult to define the system. Hence, assumptions from Chapter 4 had to be tested and C and N mass balances quantified. This allowed to evaluate the efficiency of the carbon and nitrogen sources considered and to point out improvements to increase production efficiency.

5.2. Assumptions

According with what was discussed in Chapter 4 several assumptions were considered for mass balance calculations:

- Biomass composition was considered stable throughout the process.
- Carbon from urea breakdown is assumed to be available for incorporation into biomass.
- Both carbon atoms from acetate are assumed to be available for incorporation into biomass.
- Carbon dioxide from air bubbling and pH controlling injections is consumed in amounts that are not relevant.
- The amount of dissolved CO_2 that exits the system upon harvesting is balanced by the correspondent added fresh water that is rapidly carbonated by continuous aeration.
- Both nitrogen atoms from urea are assumed to be available for incorporation into biomass.
- Ammonium in the medium originates from the metabolism of urea and must also be treated as an available nitrogen source.

5.3. Definition of the system, inlets and outlets

A black box diagram was considered to describe the photobioreactor system (

Figure 5.3.1 – Black box schematic representation of the system.). The time unit considered to perform the mass balance was one day, *i.e.*, from the morning (9 h) of day i to the morning (9 h) of the next day, i+1. Consequently, biomass (5.86C;1N), acetate (2C;0N), urea (1C;2N) and ammonium (0C;1N) in the culture medium transit from one day to the next. The carbon inlets are thus the feed and supplementation streams constituted of acetate and urea solutions. Afterwards, carbon exits the system when there is biomass harvesting or nocturnal respiration. Carbohydrate and lipid compounds are metabolized by respiration processes releasing CO_2 that cannot be reassimilated by photosynthesis in night periods resulting in cell dry weight loss (Richmond 2007; Chisti 2007; Lee et al. 1996). Similarly, nitrogen enters the system in the form of urea on the feed and supplementation streams and exits through harvesting.

The calculations performed to determine carbon and nitrogen composition of each stream are explained in detail on the flowchart in Appendix A.3.

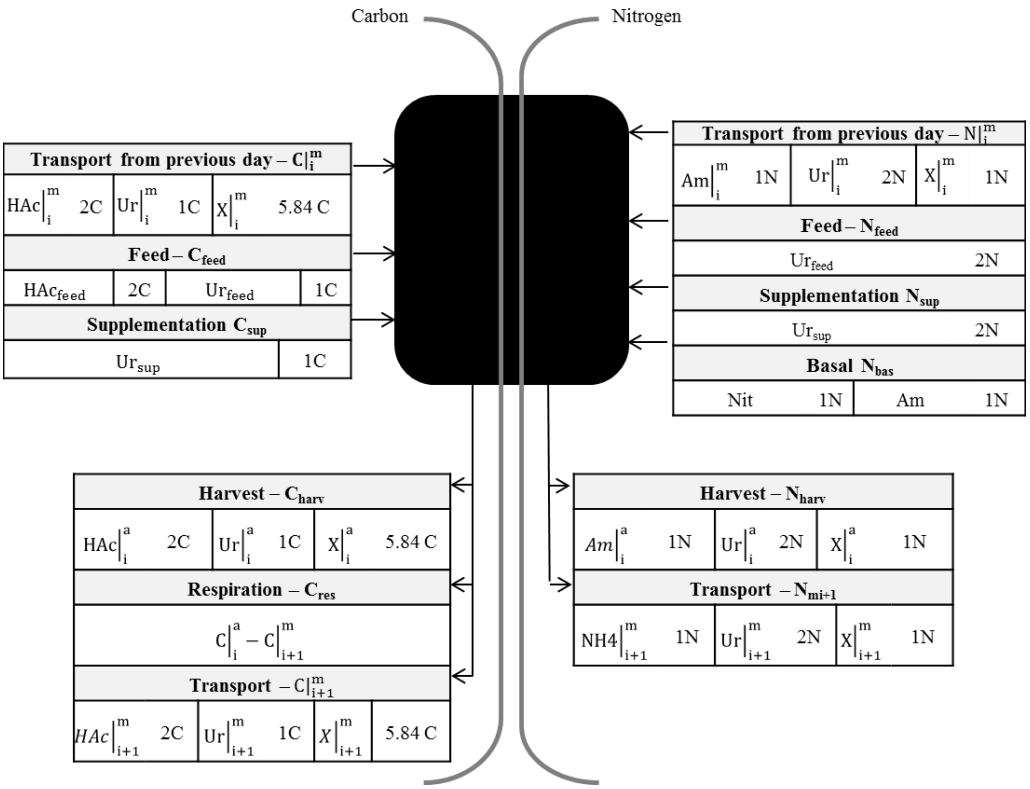

Figure 5.3.1 – Black box schematic representation of the system.

5.4. Results and discussion

The carbon and nitrogen mass balances were evaluated during the mixotrophic growth of *C. vulgaris* in PBR_S, M and L. The overall recovery factor for carbon and nitrogen was 94.4 ± 0.90 % and 76.0 ± 3.00 % with a confidence interval of 95% (n = 72). These results validate the analytical methods implemented as well as the assumptions made in Chapter 4 related to the metabolism of carbon. These results are further supported by Figure 5.4.1 that highlights the symmetry of the calculated inlets and outlets. On the other hand, the quantification of nitrogen outlets are significantly less than the correspondent inlets resulting in a lower recovery factor.

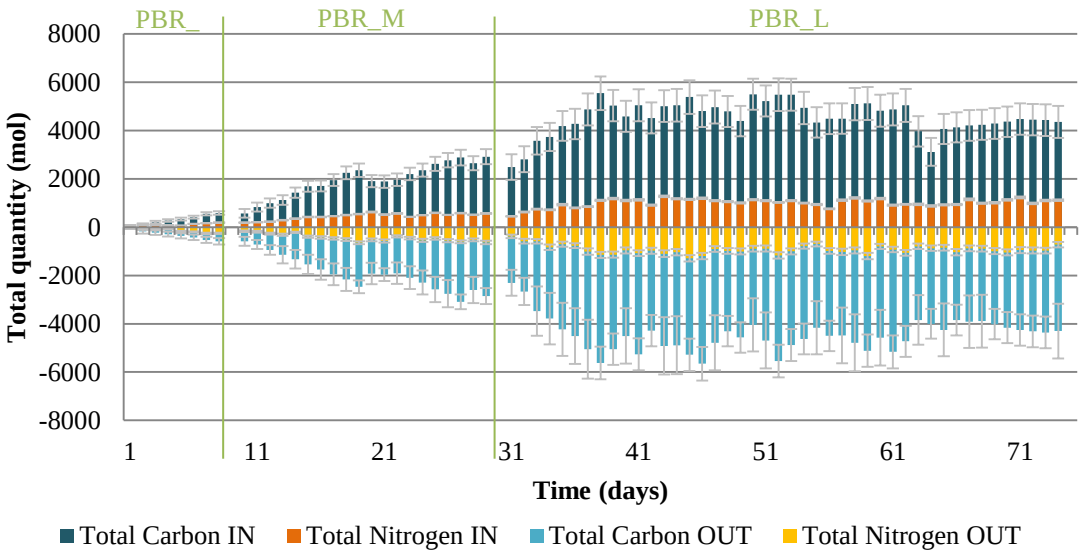

Figure 5.4.1– Graphic representation of carbon and nitrogen inlets and outlets (mol).

The significant relative error obtained relates not to the error of the analytical methods but to the one of PBR volume manipulation. Since harvesting processes are conducted by several operators by a manual method, the harvested volumes and fresh water added to restitute culture volume can vary significantly from the goal established. Hence, an arbitrary value of 10% was estimated. This is the main source of error in the mass balance and it propagates throughout all the calculations. Intermediate error values can be found in Appendix A.4.4. Lessening this error would result in narrower intervals for the calculation of accumulation which should not exist. Nevertheless, the determined model for the mass balance accounts for 97.2% and 55.6% of the existing carbon and nitrogen, respectively.

Table 5.4.1 – Carbon and nitrogen recovery factors and mass balance closure throughout the scale-up process. The closure success rate corresponds to the number of times the mass balance closes, *i.e.*, that the inlet minus the outlet stream is equal to zero within error margins.

		PBR_S	PBR_M	PBR_L	Global
Carbon recovery	$mol_{Cin}\ mol_{Cout}^{-1}$	0.92±0.04	0.96.±0.01	0.94±0.01	0.94±0.01
Nitrogen recovery	$mol_{Nin}\ mol_{Nout}^{-1}$	0.53±0.18	0.88±0.04	0.87±0.02	0.76±0.30
Carbon closure success rate	**%**	100	100	95.5	97.2
Nitrogen closure success rate	**%**	12.5	75.0	54.6	55.6

According to the data in Table 5.4.1, a noteworthy difference is observed in the ability to estimate carbon and nitrogen levels along the scale-up process. Nitrogen appears significantly underestimated in PBR_S and consequently the efficiency of the mass balance is very low at this stage of the process, only 12.5%. Nevertheless, this

underestimation of nitrogen levels is clear throughout the scale-up. Overall, the inlet-outlet deviation from zero is higher than the estimated error of this determination hence, nitrogen mass balance closure success rate is very low. This underestimation could be due to unaccounted nitrogen losses or transformations. For instance, as the photobioreactors were operated under continuous aeration in the course of this study, ammonium could evaporate given its high volatility.

5.5. Conclusions

By mass balance calculations it was possible to validate the analytical methods used for carbon estimation in the different fractions of the system as the overall recovery factors were close to 100%. Furthermore it is now possible to conclude that acetate and urea are in fact the main sources of carbon for industrial mixotrophic growth of *C. vulgaris* in the conditions of the study. The overall carbon mass balance closure success rate along with the high carbon yield discussed in Chapter 4 allows the conclusion that both acetate carbon atoms and the urea carbon are incorporated into the biomass. Hence, the sole purpose of CO_2 injections is to control the pH of the culture medium preventing it from reaching values above 8 which may be prejudicial to the cell.

Nevertheless this effectiveness was not observed for nitrogen estimation. This could be due to the lack of quantification of ammonium losses by evaporation or biological or chemical transformations that nitrogen could be subjected to. This fact could also point towards the invalidation of the assumption that stated the stability on biomass composition throughout the cultivation. As many researchers agree, biochemical composition of *C. vulgaris* is strongly dependent on growth conditions and trophic state (Chia et al. 2013; Safi et al. 2014; Kong et al. 2013; Crofcheck et al. 2013; Mandalam & Palsson 1998; Chojnacka & Marquez-Rocha 2004; Lin 2005; Pollo 2012). Therefore, stage to stage biomass elemental composition should be considered.

Model accuracy could be further increased by the reduction of the main source of error: the manual protocol for harvesting. In one alternative, the operator should record the exact culture volume that was harvested and the exact amount of water added to make up the volume. On the other hand an automated system based on the turbidity values recorded could be implemented to harvest cultures exactly to the intended concentration.

Chapter 6

6. Concluding remarks and future work

The continuous monitoring of cellular and nutrient concentrations enabled the determination of biomass productivity and substrate yields in an industrial scale production unit under mixotrophic conditions. Global elemental composition of the biomass produced was determined and verified for the later stages of scale-up.

Acetate revealed to be a super-effective carbon source as it was used by the microalgal cells with an efficiency very close to its theoretical value. Additionally, carbon yields superior to acetate yields confirms the incorporation of urea carbon into the biomass. This data, along with the mass balance calculations, shows that CO_2 injected into the culture medium can be metabolized by the cells, although at insignificant amounts, serving mainly to decrease pH levels.

On the other hand, urea metabolism results in ammonium build-up in the culture medium. The simultaneous presence of two nitrogen sources has been reported to be counterproductive to biomass growth. In fact, the low urea yield did not allow the confirmation of the incorporation of both nitrogen atoms into the biomass. Nonetheless, satisfactory nitrogen yields were obtained, indicating urea suitability as nitrogen source. Urea is also adequate as its addition does not have any effect on pH levels. In this way, routine monitoring of culture nitrogen levels should account for both urea and ammonium concentrations.

However, the high carbon and nitrogen yields discussed coupled with low biomass growth, could indicate that the production occurs close to starvation and/or the imbalance of the remaining nutrients fed to the cultures. Indeed this misbalance was confirmed in the case of phosphorus supply to the cultures. On the hand, in order to increase maximum specific growth rate and biomass productivity two strategies could be tested. One is to better suit the nutrient ratio in the feed to the cellular metabolic needs. It was possible to determine some misalignment between the carbon to nitrogen ratio consumed with that of the feed. In order to better balance these values, thorough study of biomass biochemical and elemental composition throughout the production process is needed. On the other hand the study of acetate distribution throughout the

PBR should be conducted. Supplemental feeding points on the reactor's photosynthetic zone could be tested as acetate consumption is positively correlated with light availability. This could also favour microalgal growth rather than bacterial proliferation which is a known problem of industrial mixotrophic conditions. Feed increase and adaptation to biomass growth can also be achieved by associating acetic acid addition with pH variations. Such control system has already been described (Yeh et al. 2012; Scherholz & Curtis 2013) and was implemented at Algafarm production unit at the end of this study, producing successful results which corroborate this conclusion.

A suitable mass balance model of *C. vulgaris* mixotrophic growth was obtained. It was possible to conclude that there was no considerable accumulation of carbon or nitrogen in the considered system. Thus carbon routine biomass calculations can simply be quantified assuming the complete consumption of the introduced acetate reducing the amount of analytical procedures needed. Nevertheless, in order to implement a regular base for mass balance calculations, the Algafarm record system requires further optimization. This would translate in lower error values and improved accuracy.

References

Abreu, A.P. et al., 2012. Mixotrophic cultivation of *Chlorella vulgaris* using industrial dairy waste as organic carbon source. *Bioresource technology*, 118, pp.61–6.

Borowitzka, M. a., 1999. Commercial production of microalgae: ponds, tanks, tubes and fermenters. *Journal of Biotechnology*, 70(1-3), pp.313–321.

Caspi, R., 2007. MetaCyc Pathway: urea degradation I. Available at: http://metacyc.org/META/NEW-IMAGE?type=PATHWAY&object=PWY-5703 [Accessed August 11, 2014].

Chen, C.-Y. et al., 2011. Cultivation, photobioreactor design and harvesting of microalgae for biodiesel production: A critical review. *Bioresource Technology*, 102(1), pp.71–81.

Chia, M. a, Lombardi, A.T. & Melão, M.D.G.G., 2013. Growth and biochemical composition of *Chlorella vulgaris* in different growth media. *Anais da Academia Brasileira de Ciências*, 85(4), pp.1427–38.

Chisti, Y., 2007. Biodiesel from microalgae. *Biotechnology advances*, 25(3), pp.294–306.

Chisti, Y., 2013. Constraints to commercialization of algal fuels. *Journal of biotechnology*, 167(3), pp.201–14.

Choi, W.Y. et al., 2012. Enhancement of the growth of marine microalga *Chlorella* sp . from mixotrophic perfusion cultivation for biodiesel production. *Chemical and Biochemical Engineering*, 26(3), pp.207–216.

Chojnacka, K. & Marquez-Rocha, F.-J., 2004. Kinetic and stoichiometricrelations of the energy and carbon metabolism in the culture of microalgae. *Biotechnology*, 3(1), pp.21–34.

Choochote, W., Paiboonsin, K. & Ruangpan, S., 2012. Effects of urea and light intensity on the growth of *Chlorella* sp . In *Proceedings of the 8th International Symposium on Biocontrol and Biotechnology*. pp. 127–134.

Christenson, L. & Sims, R., 2011. Production and harvesting of microalgae for wastewater treatment, biofuels, and bioproducts. *Biotechnology advances*, 29(6), pp.686–702.

Courchesne, N.M.D. et al., 2009. Enhancement of lipid production using biochemical, genetic and transcription factor engineering approaches. *Journal of biotechnology*, 141(1-2), pp.31–41.

Crofcheck, C. et al., 2013. Influence of media composition on the growth rate of *Chlorella vulgaris* and *Scenedesmus acutus* utilized for CO_2 mitigation. *Journal of Biochemical Technology*, 4(2), pp.589–594.

Filali, R. et al., 2011. Growth modeling of the green microalga *Chlorella vulgaris* in an air-lift photobioreactor. In *Preprints of the 18th IFAC World Congress*. pp. 10603–10608.

Fujita, K., 1959. The metabolism of acetate in *Chlorella* cells. *The Journal of Biochemistry*, 46(3), pp.253–268.

Goulding, K.H. & Merrett, M.J., 1966. The photometabolism of acetate by *Chlorella pyrenoidosa*. *Journal of Experimental Botany*, 17(53), pp.678–689.

Grima, E.M., Acie, F.G. & Chisti, Y., 1999. Photobioreactors : light regime , mass transfer , and scaleup. , 70, pp.231–247.

Guerra, L.T. et al., 2013. Regulatory branch points affecting protein and lipid biosynthesis in the diatom *Phaeodactylum tricornutum*. *Biomass and Bioenergy*, 59, pp.306–315.

Harun, R. et al., 2010. Bioprocess engineering of microalgae to produce a variety of consumer products. *Renewable and Sustainable Energy Reviews*, 14(3), pp.1037–1047.

Hata, J. et al., 2000. Characterization of energy conversion based on metabolic flux analysis in mixotrophic liverwort cells, *Marchantia polymorpha*. *Biochemical engineering journal*, 6(1), pp.65–74.

Hattori, A., 1960. Studies on the metabolism of urea and other nitrogenous compounds in *Chlorella ellipsoidea* - III Assimilation of urea. *Plant & Cell Physiology*, 1, pp.107–115.

Healey, F.P., 1977. Ammonium and urea uptake by some freshwater algae. *Canadian Journal of Botany*, 55(1), pp.61–69.

Heredia-Arroyo, T. et al., 2011. Mixotrophic cultivation of *Chlorella vulgaris* and its potential application for the oil accumulation from non-sugar materials. *Biomass and Bioenergy*, 35(5), pp.2245–2253.

Hodson, R.C. & Thompson, J.F., 1969. Metabolism of urea by *Chlorella vulgaris*. *Plant physiology*, 44(5), pp.691–696.

Ingraham, J.L., 2006. MetaCyc Pathway: superpathway of acetate utilization and formation. Available at: http://metacyc.org/META/NEW-IMAGE?type=PATHWAY&object=ACETATEUTIL-PWY [Accessed August 10, 2014].

Johnson, X. & Alric, J., 2013. Central carbon metabolism and electron transport in *Chlamydomonas reinhardtii*: metabolic constraints for carbon partitioning between oil and starch. *Eukaryotic cell*, 12(6), pp.776–793.

Kim, S. et al., 2013. Growth rate, organic carbon and nutrient removal rates of *Chlorella sorokiniana* in autotrophic, heterotrophic and mixotrophic conditions. *Bioresource technology*, 144, pp.8–13.

Kong, W. et al., 2013. Effect of glycerol and glucose on the enhancement of biomass , lipid and soluble carbohydrate production by *Chlorella vulgaris* in mixotrophic culture. *Food Technology and Biotechnology*, 51(1), pp.62–69.

Lee, Y.-K., 2001. Microalgal mass culture systems and methods : Their limitation and potential. *Journal of Applied Phycology*, 13, pp.307–315.

Lee, Y.-K. et al., 1996. Mixotrophic growth of *Chlorella sorokiniana* in outdoor enclosed photobioreactor. *Journal of Applied Phycology*, 8, pp.163–169.

Liang, Y., Sarkany, N. & Cui, Y., 2009. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnology Letters*, 31, pp.1043–1049.

Lin, L., 2005. *Chlorella:* Its Ecology, Structure, Cultivation, Bioprocess and Application, Taipei, Taiwan: Yi Hsien Publishing Company.

Lin, L. & Chen, T., 1994. Factors affecting the mixotrophic maximum growth of *Chlorella pyrenoidosa*. *Journal of the Chinese Agricultural Chemical Society*, 32(l), pp.91–102.

Mandalam, R.K. & Palsson, B.O., 1998. Elemental balancing of biomass and medium composition enhances growth capacity in high-density *Chlorella vulgaris* cultures. *Biotechnology and bioengineering*, 59(5), pp.605–611.

Markou, G., Vandamme, D. & Muylaert, K., 2014. Microalgal and cyanobacterial cultivation: The supply of nutrients. *Water Research*.

Martinez, F. & Orus, M.I., 1991. Interactions between glucose and inorganic carbon metabolism in *Chlorella vulgaris* Strain UAM 1011. *Plant Physiology*, 95, pp.1150–1155.

Merrett, M.J. & Goulding, K.H., 1968. The glycollate pathway during the photoassimilation of acetate by *Chlorella*. *Planta*, 80, pp.321–327.

Molina Grima, E. et al., 2003. Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnology advances*, 20(7-8), pp.491–515.

Molloy, C.J. & Syrett, P.J., 1988. Interrelationships between uptake of urea and uptake of sammonium by microalgae. *Journal of Experimental Marine Biology and Ecology*, 118, pp.85–95.

Muthuraj, M. et al., 2013. Flux balance analysis of *Chlorella* sp. FC2 IITG under photoautotrophic and heterotrophic growth conditions. *Photosynthesis research*, 118(1-2), pp.167–79.

Neilson, A.H. & Lewin, R.A., 1974. The uptake and utilization of organic carbon by algae : an essayin comparative biochemistry. *Phycologia*, 13(3), pp.227–264.

Ogawa, T. & Aiba, S., 1981. Bioenergetic analysis of mixotrophic growth in *Chlorella vulgaris* and *Scenedesmus acutus*. *Biotechnology and bioengineering*, 23(5), pp.1121–1132.

Pagnanelli, F. et al., 2014. Mixotrophic growth of *Chlorella vulgaris* and *Nannochloropsis oculata* : influence of glucose and nitrate concentration. *Journal of Chemical Technology & Biotechnology*, 89(5), pp.652–661.

Perez-Garcia, O. et al., 2011. Heterotrophic cultures of microalgae: metabolism and potential products. *Water research*, 45(1), pp.11–36.

Pollo, C.A., 2012. Evaluation of mass and energy balances in the combined microalgae growth-digestion process. Valladolid University.

Quinn, J., de Winter, L. & Bradley, T., 2011. Microalgae bulk growth model with application to industrial scale systems. *Bioresource technology*, 102(8), pp.5083–92.

Rawat, I. et al., 2013. Biodiesel from microalgae: A critical evaluation from laboratory to large scale production. *Applied Energy*, 103, pp.444–467.

Richmond, A. ed., 2007. Handbook of Microalgal Culture: Biotechnology and Applied Phycology, Oxford, UK: Blackwell Publishing Ltd.

Safi, C. et al., 2014. Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. *Renewable and Sustainable Energy Reviews*, 35, pp.265–278.

Scherholz, M.L. & Curtis, W.R., 2013. Achieving pH control in microalgal cultures through fed-batch addition of stoichiometrically-balanced growth media. *BMC biotechnology*, 13(1), pp.13-39.

Sforza, E. et al., 2012. Excess CO_2 supply inhibits mixotrophic growth of *Chlorella protothecoides* and *Nannochloropsis salina*. *Bioresource technology*, 104, pp.523–529.

Spolaore, P. et al., 2006. Commercial applications of microalgae. *Journal of bioscience and bioengineering*, 101(2), pp.87–96.

Syrett, P.J., Bocks, S.M. & Merrett, M.J., 1964. The assimilation of acetate by *Chlorella vulgaris*. *Journal of Experimental Botany*, 15(1), pp.35–47.

Watt, G.W. & Chrisp, J.D., 1954. Spectrophotometric method for determination of urea. *Analytical Chemistry*, 16(3), pp.452–453.

Yang, C., Hua, Q. & Shimizu, K., 2000. Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions. *Biochemical engineering journal*, 6(2), pp.87–102.

Yang, J. et al., 2011. Mathematical model of *Chlorella minutissima* UTEX2341 growth and lipid production under photoheterotrophic fermentations conditions. *Bioresource technology*, 102(3), pp.3077–3082.

Yeh, K.-L. & Chang, J.-S., 2012. Effects of cultivation conditions and media composition on cell growth and lipid productivity of indigenous microalga *Chlorella vulgaris* ESP-31. *Bioresource technology*, 105, pp.120–7.

Yeh, K.-L., Chen, C.-Y. & Chang, J.-S., 2012. pH-stat photoheterotrophic cultivation of indigenous *Chlorella vulgaris* ESP-31 for biomass and lipid production using acetic acid as the carbon source. *Biochemical Engineering Journal*, 64, pp.1–7.

Appendix

A Appendix

A.1 Calibration curves

A.1.1 Turbidity (NTU) *versus* dry weight (g L^{-1})

The calibration curve of turbidity *versus* dry weight was obtained using experimental points from all photobioreactor types in the period between 01/10/2013 and 09/07/2014. It is of importance to note that the determined correlation is only valid in the range of values presented.

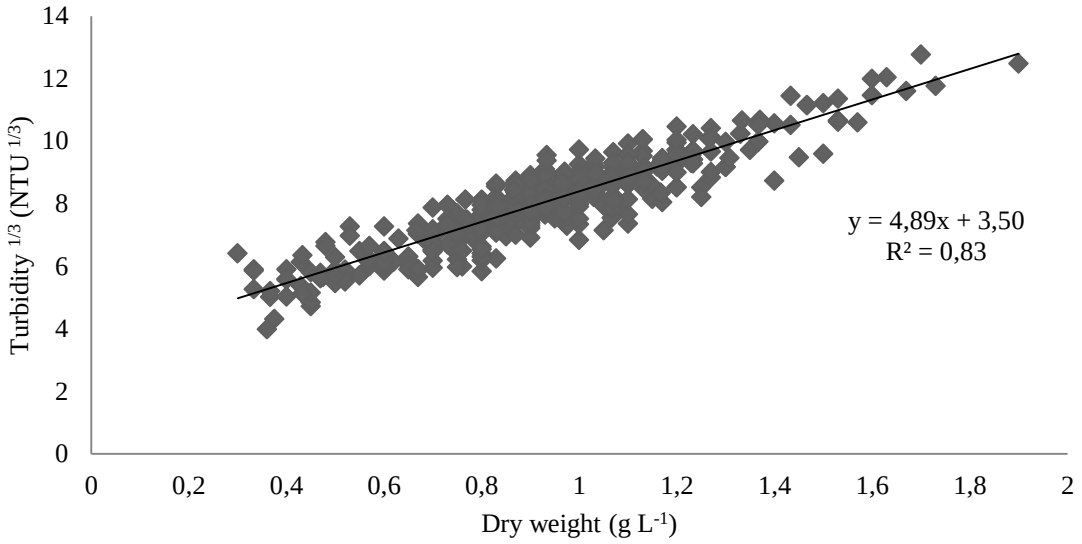

Figure A.1.1.1 – Calibration curve for biomass quantification of large-scale mixotrophically grown *C. vulgaris*

Table A.1.1.1– Specific parameters of the turbidity *versus* dry weight calibration curve.

Slope, a	-	4.89
Y-intersect, b	-	3.50
Number of points, n	-	485
Correlation coefficient, R	-	0.91
Standard deviation in the residuals, Syx	-	0.55
Detection limit	g L^{-1}	0.06
Quantification limit	g L^{-1}	0.20
Precision, %CV	-	-
Accuracy, Er	-	-

A.1.2 **Absorbance (418 nm) *versus* urea concentration (mmol L^{-1})**

The calibration curve of the absorbance at 418 nm *versus* urea concentration (mM) used had been previously obtained.

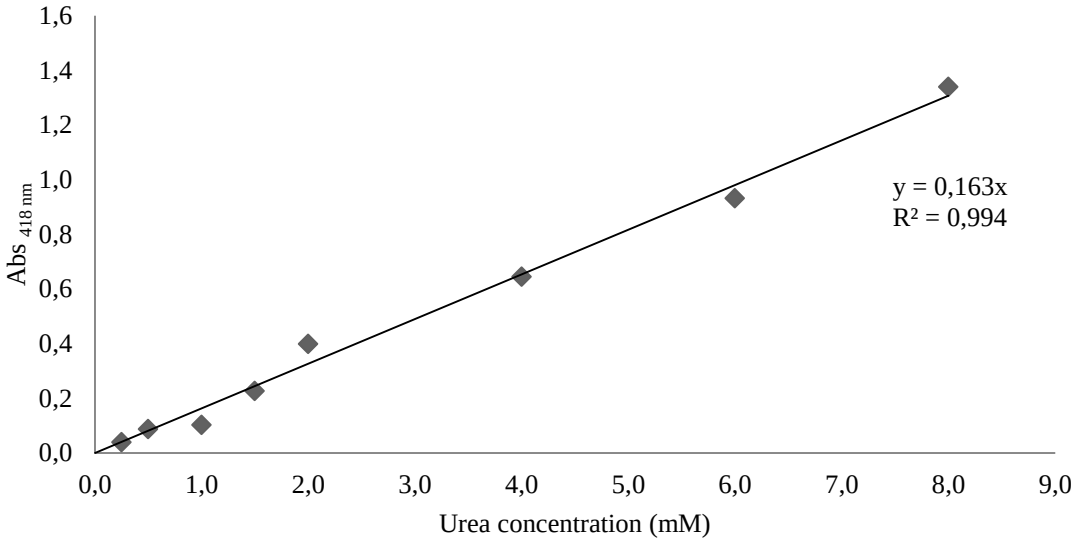

Figure A.1.2.1 – Calibration curve for urea quantification

Table A.1.2.1 – Specific parameters of the $Abs_{418\ nm}$ *versus* urea concentration calibration curve.

Slope, a	-	0.163
Y-intersect, b	-	0
Number of points, n	-	9
Correlation coefficient, R	-	0.996
Standard deviation in the residuals, Syx	-	0.0272
Detection limit	mmol L^{-1}	0.18
Quantification limit	mmol L^{-1}	0.62
Precision, %CV	-	1,05
Accuracy, Er	-	20.8%

A.1.3 **Absorbance (697 nm) *versus* ammonium concentration (mmol L^{-1})**

The calibration curve of absorbance at 697 nm *versus* ammonium concentration (mM) was obtained.

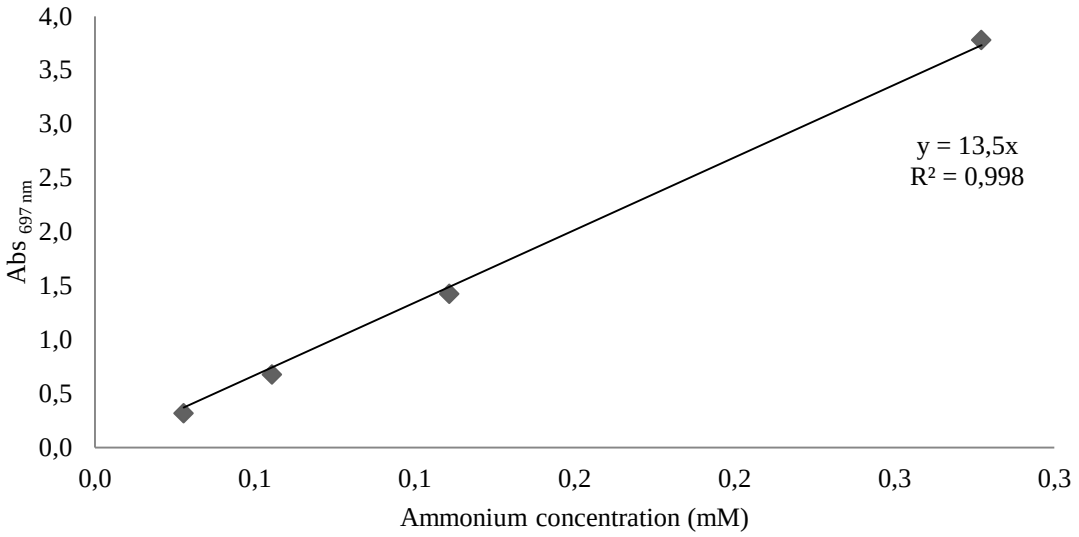

Figure A.1.3.1– Calibration curve for ammonium quantification.

Table A.1.3.1 – Specific parameters of the Abs_{697nm} *versus* ammonium concentration calibration curve.

Slope, a	-	13.5
Y-intersect, b	-	0
Number of points, n	-	4
Correlation coefficient, R	-	0.999
Standard deviation in the residuals, Syx	-	0.135
Detection limit	mmol L^{-1}	0.023
Quantification limit	mmol L^{-1}	0.075
Precision, %CV	-	-
Accuracy, Er	-	-

A.1.4 **Absorbance (340 nm) *versus* acetate concentration (mmol L^{-1})**

The calibration curve of absorbance at 340 nm *versus* acetate concentration (mM) used was obtained using the method by Megazyme, K-ACETRM 07/12.

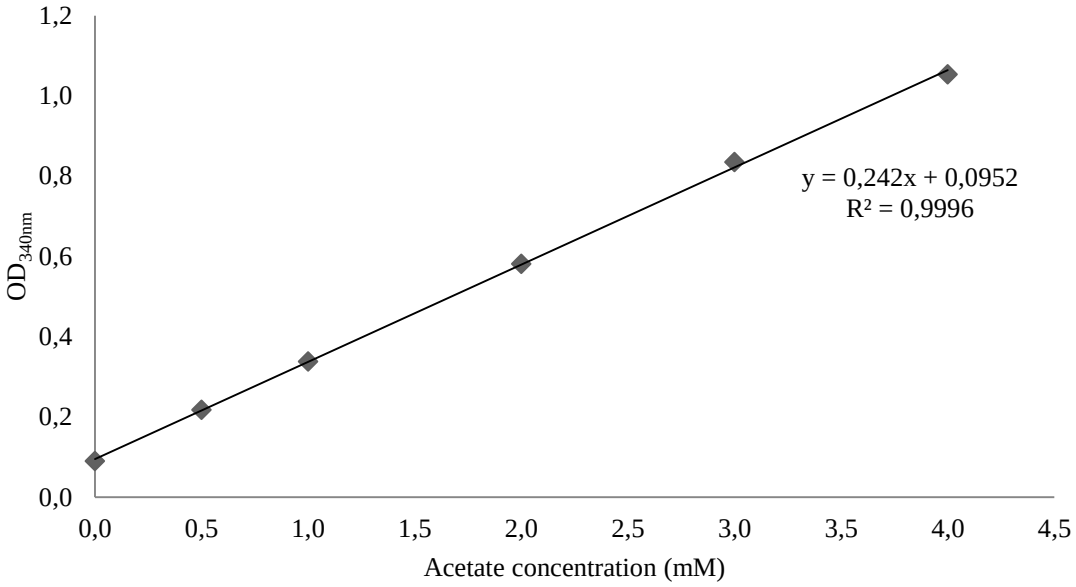

Figure A.1.4.1 – Calibration curve for acetate quantification.

Table A.1.4.1 – Specific parameters of the Abs_{340nm} versus acetate concentration calibration curve.

Slope, a	-	0.242
Y-intersect, b	-	0.0952
Number of points, n	-	6
Correlation coefficient, R	-	0.9998
Standard deviation in the residuals, Syx	-	0.0088
Detection limit	mmol L^{-1}	0.07
Quantification limit	mmol L^{-1}	0.24
Precision, %CV	-	2.82
Accuracy, Er	-	2.06%

A.2 Estimation of the elemental composition of biomass

A.2.1 **Biochemical analysis**

Dried *C. vulgaris* biomass from Algafarm batches A, B, C and D from the PBR_L was analysed by Silliker, Inc. Results from biochemical analysis of main compounds are summarized in Table A.2.1.1. Full biochemical analysis of batch A is presented in Table A.2.1.2.

Table A.2.1.1 Results of partial biochemical analysis to mixotrophic *Chlorella vulgaris*.

	A	B	C	D.1	D.2
	26-03-2014	13-05-2014	13-05-2014	13-05-2014	13-05-2014
	% w/w	% w/w	% w/w	% w/w	% w/w
Carbohydrates	4.2	1.4	2.5	3.7	4.4
Dietary fibre	18.2	17.8	18.7	18.8	23
Fat	9.87	9.04	9.99	9.87	10.6
Protein	56.3	58.15	55.93	55.52	50.28
Ash	7.31	9.17	8.5	7.54	6.76
Moisture	4.1	4.4	4.4	4.6	5
Energy value	367	355	361	363	
Water activity	<0.25	<0.25	<0.25	<0.25	<0.25

Table A.2.1.2 Results of complete biochemical analysis to mixotrophic *Chlorella vulgaris* batch LA.

	A	
	26-03-2014	
	Molecular formula	% w/w
Carbohydrates	$C_6H_{12}O_6$	4.2
Dietary fibre	$C_6H_{10}O_5$	18.2
Palmitic acid	$C_{16}H_{32}O_2$	1.77
Palmitoleic acid	$C_{16}H_{30}O_2$	0.75
Stearic acid	$C_{18}H_{36}O_2$	0.16
Oleic acid	$C_{18}H_{34}O_2$	0.47
Linoleic acid	$C_{18}H_{32}O_2$	1.79
Alpha-linolenic acid	$C_{18}H_{33}O_2$	2.51
Octadecenoic acid	$C_{18}H_{34}O_2$	2.08
Glutamic Acid	$C_5H_{10}N_2O_3$	9.472
Methionine	$C_5H_{11}NO_2S$	0.055
Proline	$C_5H_9NO_2$	3.594
Hidroxiproline	C_5H_9NO3	-
Lysine	$C_6H_{14}N_2O_2$	4.924
Tyrosine	$C_9H_{11}NO_3$	1.744
Phenylalanine	$C_9H_{11}NO_2$	2.447

Serine	$C_3H_7NO_3$	2.233
Hystidine	$C_6H_9N_3O_2$	0.894
Glycine	$C_5H_5NO_2$	2.779
Threonine	$C_4H_9NO_3$	2.549
Arginine	$C_6H_{14}N_4O_2$	3.758
Alanine	C_3H_4NO	5.335
Valine	$C_5H_{11}NO_2$	2.819
Aspartic acid	$C_4H_7NO_4$	5.525
Tryptophan	$C_{11}H_{12}N_2O_2$	1.4
Isoleucine	C_6H_7NO	1.775
Leucine	$C_6H_{13}NO_2$	3.832
Cistine	$C_6H_{12}N_2O_4$	0.000
Asparagine	$C_4H_8N_2O_3$	0.376
Glutamine	$C_5H_{10}N_2O_3$	0.149

A.2.2 **Elemental formula estimation**

From this data, biomass elemental composition ($C_aN_bO_cH_dS_e$) was obtained by Eq. A.2.2.1 to Eq. A.2.2.5.

$$a = \sum_i^n \%\, w/w_i \times \frac{n_C}{M_i} \quad \text{(A.2.2.1)}$$

$$b = \sum_i^n \%\, w/w_i \times \frac{n_N}{M_i} \quad \text{(A.2.2.2)}$$

$$c = \sum_i^n \%\, w/w_i \times \frac{n_O}{M_i} \quad \text{(A.2.2.3)}$$

$$d = \sum_i^n \%\, w/w_i \times \frac{n_H}{M_i} \quad \text{(A.2.2.4)}$$

$$e = \sum_i^n \%\, w/w_i \times \frac{n_S}{M_i} \quad \text{(A.2.2.5)}$$

Where a, b, c, d and e are the stoichiometric coefficients of carbon, nitrogen, oxygen, hydrogen and sulphur, respectively. n_α represents the stoichiometric coefficient of atom α in the macromolecule considered, M_i.

As discussed in Section 4.2.5, fat and protein content of batches with only partial results were segmented considering the distribution equal to that of the detailed profile of batch A. The mean value of the stoichiometric coefficients was considered for the elemental composition: $C_{5.84}N_{1.00}O_{2.85}H_{10.39}S_{0.001}$.

Carbon and nitrogen conversion factors can now be calculated according to the biomass elemental formula (Eq A.2.2.6).

$$f_\alpha = \frac{n_\alpha}{M_X} \quad mol_\alpha \; g_X^{-1} \qquad \text{(A.2.2.6)}$$

Where n_α is the stoichiometric coefficient of atom and M_X the biomass molecular weight considering the composition $C_{5.84}N_{1.00}O_{2.85}H_{10.39}S_{0.001}$.

A.3 Mass balance calculations

A flowchart was designed to illustrate the calculations made for the mass balance of carbon (Figure A.3.1) and nitrogen (Figure A.3.2).

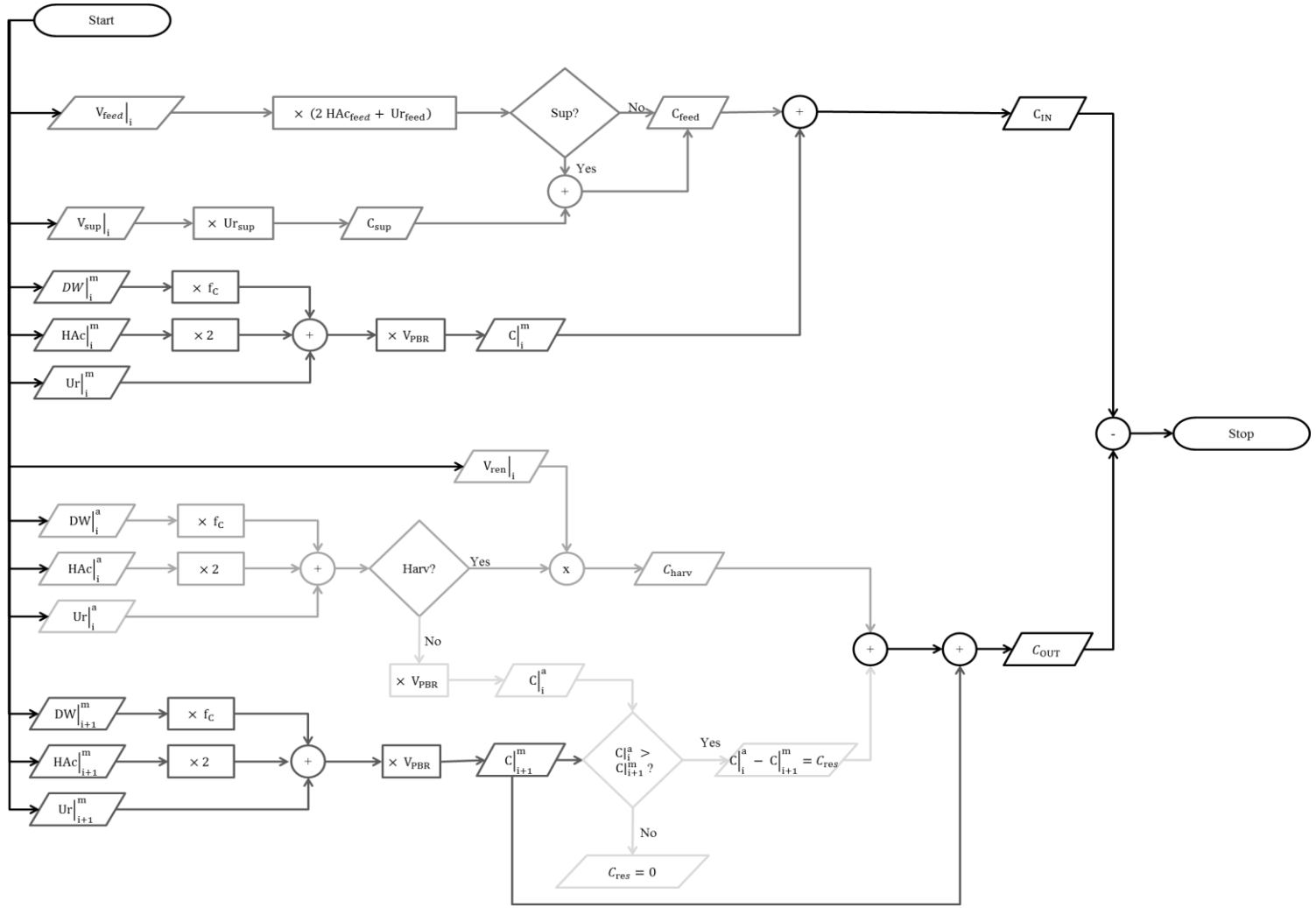

Figure A.3.11 – Flowchart of carbon mass balance; feed streams are represented in (▬), harvesting in (▬), respiration in (▬) and day to day transport streams in (▬).

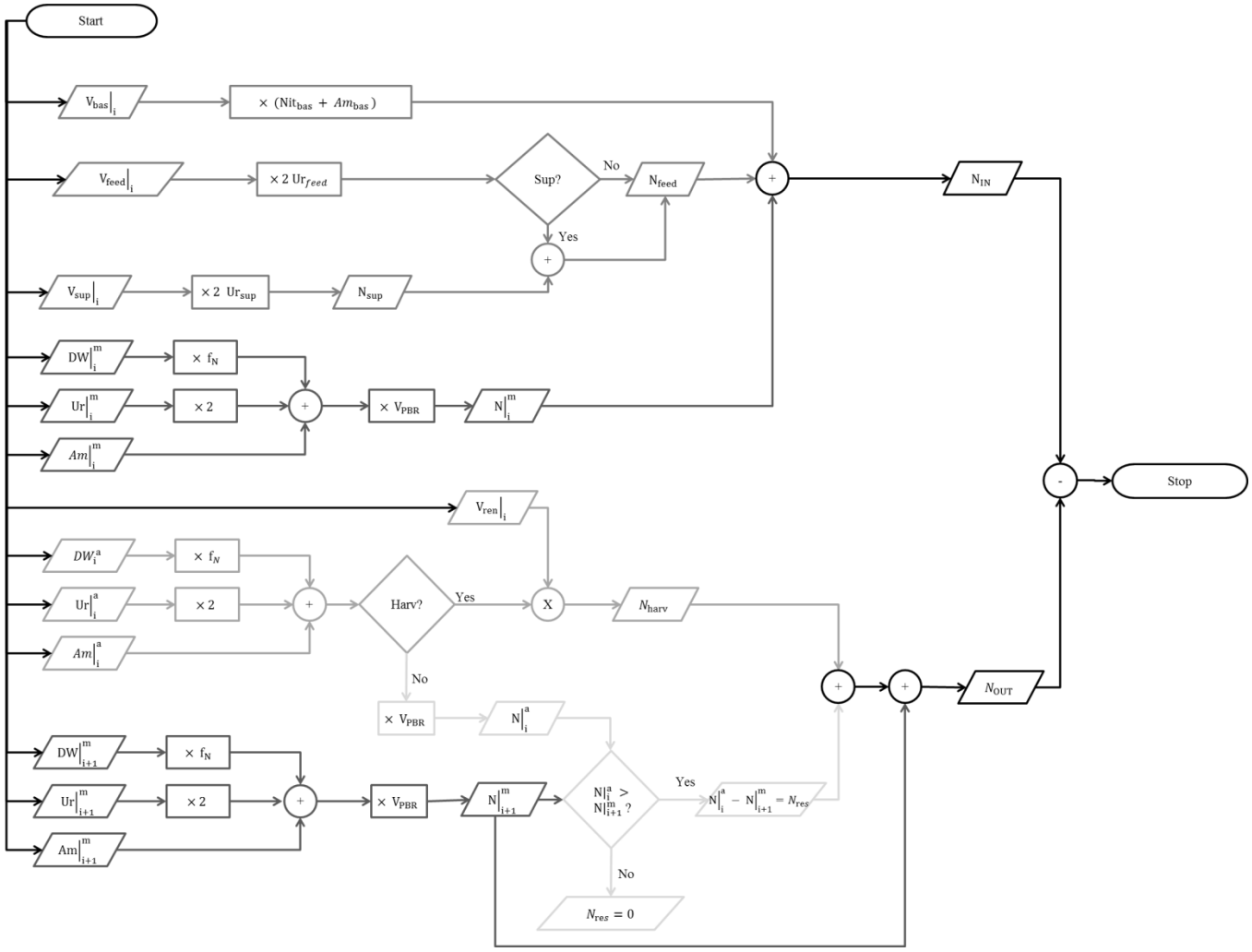

Figure A.3.2 – Flowchart of nitrogen mass balance. Feed streams are represented in (▬), harvesting in (▬), respiration in (▬) and day to day transport streams in (▬).

A.4 Error estimation

A.4.1 **Analytical methods**

Error values from analytical methods were estimated according to Eq. A.4.1.1

$$S_{x_0} = \frac{S_{yx}}{a}\sqrt{\frac{1}{m}+\frac{1}{n}+\frac{(y_0-\bar{y})^2}{a^2\sum(x_i-\bar{x})^2}} \quad \text{(A.4.1.1)}$$

Where S_{yx} is the standard deviation of the residuals, a the slope, n the number of points, x_i the x-values of the calibration curve, $\bar{y}$ and $\bar{x}$ the mean values of y and x obtained in the calibration curve, m the number of replicas in the experimental determination and y_0 the experimental value obtained.

Average relative errors were calculated for all analytical methods.

Table A.4.1.1 – Average values of relative errors in analytical methods.

	Dry weight	**Urea**	**Ammonium**	**Acetate**
Average	13%	10%	1%	9%

A.4.2 **Estimated values**

Relative error values of 10% were attributed to all processes conducted by several operators. Hence, V_{feed}, V_{bas}, V_{harv} and V_{PBR} have an estimated error of ± 10%.

A.4.3 **Error propagation**

Propagation of errors was calculated according to Eq. A.4.3.1 and Eq. A.4.3.2 for sum and multiplication operations, respectively.

$$\Delta z = \sqrt{\Delta x^2 + \Delta y^2 + \cdots} \quad \text{(A.4.3.1)}$$

$$\frac{\Delta z}{z} = \sqrt{\left(\frac{\Delta x}{x}\right)^2 + \left(\frac{\Delta z}{z}\right)^2 + \cdots} \quad \text{(A.4.3.2)}$$

Where $\Delta\alpha$ is the absolute error associated to the value α.

A.4.4 **Intermediate errors**

Relative errors of intermediate calculations are presented below.

Table A.4.4.1 – Mean and standard deviation values of relative errors of calculation.

	Carbon		**Nitrogen**	
	Average	Standard deviation	Average	Standard deviation
Feed medium	10%	-	10%	-
Supplementation	11%	-	11%	-
Basal medium	-	-	5%	-
$\boldsymbol{\alpha\vert_i^m}$	16%	5%	14%	2%
Total α IN	14%	3%	3%	1%
Harvest	14%	4%	14%	2%
Respiration*	1282%	3539%	-	-
$\boldsymbol{\alpha\vert_{i+1}^m}$	17%	5%	14%	2%
Total α OUT	22%	7%	13%	2%
α IN - α OUT	1547%	2739%	517%	2207%