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***OIL FROM MICROALGAE:
SPECIES SELECTION, PHOTOBIOREACTOR DESIGN
AND PROCESS OPTIMIZATION***

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RIASSUNTO

La produzione di biocarburanti da biomassa sta suscitando un vivo interesse a livello internazionale e, in questo contesto, l'olio derivato da microalghe sembra essere l'unica tecnologia potenzialmente in grado di supportare la richiesta energetica di combustibili liquidi per autotrazione e sostituire, nel lungo termine, i carburanti da fonti fossili (Chisti, 2008). La produzione di biomassa algale per via fotosintetica, inoltre, ha dei notevoli vantaggi dal punto di vista ambientale, contribuendo alla diminuzione dell'immissione dei gas serra in atmosfera e all'eliminazione di sali di ammonio e fosforo dalle acque di scarico.

Per microalghe s'intendono tutti gli organismi unicellulari, o di piccole dimensioni, che possiedono la clorofilla A (grazie alla quale operano fotosintesi) e che presentano un tallo non differenziato in radice-fusto-foglia. In questa classificazione vengono inglobati generalmente anche i cianobatteri, sebbene siano organismi procarioti (Mata et al., 2010). L'interesse verso questi organismi nasce, storicamente, dalla potenzialità di utilizzo per la produzione di biomassa a scopo alimentare, per la nutrizione animale e per la produzione di composti chimici, sfruttando l'energia solare. La fotosintesi, infatti, è un processo di conversione di composti inorganici e energia solare in materia organica. Gli organismi in grado di effettuare tali reazioni vengono definiti fotoautotrofi. La fotosintesi ossigenica, in particolare, può essere definita come una serie di reazioni di ossido-riduzione, nelle quali l'anidride carbonica e l'acqua sono trasformate in carboidrati e ossigeno. In presenza di macronutrienti (principalmente nitrati e fosfati, e una fonte di carbonio) e micronutrienti (principalmente metalli, utilizzati come cofattori) le microalghe sono in grado di riprodursi, generalmente mediante divisione asessuata, con una velocità notevolmente maggiore rispetto alle piante superiori terrestri. Ciò rende le microalghe particolarmente adatte alla coltivazione su larga scala per l'assorbimento della CO₂ atmosferica, per la produzione di biocombustibili, per la depurazione di reflui civili e agro-zootecnici e per la produzione di biomolecole. Microalghe di varie specie vengono già prodotte a livello commerciale in molti Paesi e utilizzate, in genere, per ottenere integratori alimentari, mangimi, pigmenti, acidi grassi, ω3, biomasse per acquacoltura e per il trattamento di reflui.

E' stato osservato che alcune specie di microalghe sono in grado di accumulare grandi quantità di lipidi, che possono essere estratti ed utilizzati come oli vegetali, al pari degli oli estratti dai semi delle piante superiori. I vantaggi dell'utilizzo delle microalghe sono legati al fatto che questi organismi presentano elevate velocità di crescita, e possono essere coltivati massivamente in fotobioreattori, senza bisogno di terreni coltivabili ed eliminando, di conseguenza, il problema della competizione con risorse agricole destinate ad uso alimentare. La coltivazione di microalghe legata alla produzione di biodiesel, quindi, è una tecnologia che potrebbe avere un elevato potenziale di sviluppo, consentendo una netta riduzione delle emissioni di CO₂ rispetto all'uso di combustibili fossili, senza sottrarre risorse alle coltivazioni terrestri per fini alimentari. Inoltre, dopo l'estrazione di biocombustibile, la biomassa microalgale residua potrebbe ancora essere impiegata per l'estrazione di biomolecole di interesse commerciale, per la produzione di biogas o per scopi energetici (Chisti, 2008).

La scelta dei sistemi di coltura di questo tipo su larga scala è tuttora oggetto di studio (Mata et al., 2010; Grobbelaar, 2010; Ho et al., 2011). Tali sistemi si distinguono in due categorie principali: sistemi aperti (*open ponds*) e sistemi chiusi (fotobioreattori). Costituiti da circuiti generalmente tubolari o a pannello, i fotobioreattori presentano un grado di complessità decisamente maggiore rispetto ai sistemi aperti ma consentono uno stretto controllo dei parametri chimico-fisici e biologici della coltura e una migliore resa produttiva. Le maggiori criticità sono da imputare al controllo della temperatura e al rischio di accumulo dell'ossigeno prodotto per fotosintesi, che richiede sistemi di eliminazione di questo gas. Questi problemi limitano le dimensioni dei fotobioreattori, che attualmente sono costituiti principalmente da serpentine di lunghezza non elevata e volumi limitati. Di conseguenza il costo della produzione di microalghe è piuttosto elevato, per cui le applicazioni rimangono limitate alle sole colture massive di elevata purezza, necessarie per l'estrazione di biomolecole di alto valore commerciale o per inoculi di colture in sistemi aperti.

La ricerca internazionale sta puntando sempre più l'attenzione su questa tecnologia (Pittman et al., 2011; Perez-Garcia et al., 2011; Ho et al., 2011), ma il mondo delle microalghe è tuttavia molto vasto; è, quindi, necessario scegliere accuratamente la specie più adatta dal punto di vista della velocità di riproduzione e del contenuto di lipidi su massa secca. Inoltre, è necessario tenere conto delle condizioni di coltura al fine di ottimizzare la crescita e l'accumulo di lipidi (Amaro et al., 2011).

Questa tesi di dottorato si è occupata della scelta delle specie microalgali più promettenti, oggetto di interesse internazionale, e dell'ottimizzazione delle condizioni di coltura, volti alla progettazione di un fotobioreattore.

A tal scopo, la parte preliminare di questo progetto di ricerca ha visto la messa a punto delle principali tecniche di coltura e analisi della crescita microalgale.

Una volta messe a punto le metodiche, si è proceduto con uno *screening* delle specie più interessanti nell'ottica della produzione di olio vegetale, scelte dopo consultazione della bibliografia disponibile.

Nannochloropsis salina, una specie marina, sembra essere la specie più adatta per la produzione di olio, mostrando la miglior combinazione di velocità cinetiche di crescita (circa 0,5 giorni⁻¹) e di contenuto di lipidi. Infatti, il contenuto lipidico dell'alga cambia durante la fase di crescita, mostrando un significativo accumulo di lipidi in fase stazionaria corrispondente al circa 69% del peso secco. Ciò è probabilmente determinato dall'accumulo di lipidi come materiale di riserva quando le condizioni di crescita risultano limitanti. Considerando l'elevato contenuto di lipidi, questa specie mostra delle reali potenzialità dal punto di vista applicativo, e quindi è stata sottoposta ad ulteriori esperimenti di ottimizzazione delle condizioni di coltura, variando le concentrazioni di nutrienti nel mezzo di crescita e allestendo apparecchiature sperimentali in grado di fornire concentrazioni di anidride carbonica maggiori rispetto a quelle atmosferiche, risultate limitanti per una produzione significativa di biomassa. Gli esperimenti, quindi, sono stati condotti insufflando nella coltura aria arricchita al 5% di CO₂ in modo tale che non fosse limitante per la crescita. Altri esperimenti sono stati finalizzati a comprendere se la concentrazione di azoto nel terreno di coltivazione fosse limitante per *N. salina*. Sono state quindi testate diverse concentrazioni di nitrato di sodio mantenendo la concentrazione di CO₂ del 5% nell'aria insufflata. I risultati hanno mostrato che la CO₂ presente in aria è limitante per la crescita. Inoltre, sebbene con il 5% la coltura raggiunga concentrazioni più elevate in fase stazionaria rispetto alla coltura insufflata con semplice aria, il nutrimento veramente limitante è chiaramente l'azoto. Infatti, in presenza di 1,5 g/L di NaNO₃ (circa 20 volte la concentrazione normalmente utilizzata nei terreni di coltura) la concentrazione cellulare in fase stazionaria arriva ad un valore 4 volte maggiore di quello misurato negli altri casi.

Quando l'azoto è presente in eccesso, tuttavia, il contenuto di lipidi rimane basso. Questo sembra suggerire che, effettivamente, l'aumento di lipidi sia determinato dalla carenza di azoto. Infatti, cellule raccolte per centrifugazione e risospese in un terreno povero in azoto, mostrano un aumento di fluorescenza corrispondente al 63±1% di lipidi su peso secco. Questi dati dimostrano che la deficienza di azoto in *N. salina* è responsabile dell'accumulo di lipidi. È noto, infatti, che la composizione biochimica delle microalghe può essere modificata attraverso manipolazioni ambientali, inclusa la disponibilità dei nutrienti. A questo scopo, per specifiche applicazioni, alcuni nutrienti vengono somministrati in concentrazioni limitanti. In particolare, il contenuto di lipidi in alcune alghe può variare come risultato di cambiamenti nelle condizioni di crescita o nelle caratteristiche del mezzo di coltura (Rodolfi et al., 2009; Converti et al., 2009). Il più efficiente approccio per aumentare il contenuto di lipidi nella alghe sembra essere la deficienza di azoto. In queste condizioni, la produttività della coltura è

generalmente ridotta, se messa a confronto con le condizioni di nutrienti in eccesso (Rodolfi et al., 2009). Infatti, la deprivazione di azoto è generalmente associata ad una riduzione nella resa di biomassa ed ad una diminuzione della crescita.

Questo spiega i risultati sperimentali ottenuti, in cui l'elevata concentrazione di nitrati, nella prima fase, stimola la crescita di biomassa, probabilmente stimolando la sintesi e l'accumulo di proteine, mentre, durante la fase limitante in azoto le alghe cominciano ad accumulare lipidi, e si registra un netto aumento della concentrazione di massa secca per cellula, fino ad una concentrazione complessiva di 4.05 g/L DW.

Dal punto di vista industriale, quindi, la strategia vincente è probabilmente un approccio a due step, sperimentata con successo anche da (Rodolfi et al., 2009), con una prima fase di produzione di biomassa in terreno con sufficiente concentrazione di nutrienti (N-sufficient phase), seguita da un'induzione di accumulo lipidico attraverso deprivazione d'azoto (N-starved phase).

In questa tesi è stata, inoltre, presa in considerazione la crescita mixotrofa. In colture algali a scala industriale, infatti, la crescita in fotoautotrofia potrebbe presentare alcuni limiti, legati soprattutto alla produttività. Ciò è dovuto sia alla scarsa penetrazione della luce in colture su larga scala, che è inversamente proporzionale alla densità cellulare, sia ai limiti intrinseci dell'efficienza fotosintetica delle microalghe. Per incrementare la produttività, una possibile strategia è crescere le colture in mixotrofia, esponendo quindi le alghe alla luce, ma fornendo anche un substrato organico che migliori la velocità cinetica di crescita e la resa in biomassa. L'obiettivo di questa parte del progetto di ricerca è stato di studiare gli effetti sulla crescita algale di diversi substrati organici, e di ottimizzare le condizioni di crescita mixotrofa. A tale scopo, *N. salina* e altre specie interessanti sono state sottoposte ad uno studio più accurato delle condizioni di crescita mixotrofa. Successivamente, è stata testata la capacità delle microalghe di utilizzare substrati organici durante i periodi di buio, in curve di crescita soggette a cicli di illuminazione giorno-notte.

In generale è possibile concludere che le microalghe prese in considerazione sono in grado di aumentare le loro *performance* di crescita in presenza di fonti di carbonio organico addizionato al mezzo di coltura, rispetto alla sola crescita fotoautotrofa, anche se le diverse specie rispondono in modo diverso alla presenza dei vari substrati. Questa osservazione è vera però soltanto per le colture cresciute in condizioni di CO₂ atmosferica. Quando la CO₂ non è limitante, invece, l'aggiunta del substrato organico non solo non migliora le velocità di crescita, ma sembra inibire la crescita algale. Una possibile spiegazione del fenomeno è che, in una situazione in cui la CO₂ è presente in eccesso, le microalghe preferiscono seguire la via fotosintetica, non consumando il substrato organico che, rimanendo nel mezzo di coltura, dà luogo a fenomeni di inibizione. In proposito, alcuni lavori riportano dati di inibizione della

crescita in presenza di alte concentrazioni di substrati organici (Lee et al., 2007). Risultati più interessanti invece riguardano il contenuto di lipidi, che aumenta in presenza del substrato organico. Il ruolo determinante della CO₂ è stato dimostrato in esperimenti in mixotrofia, in cui l'apporto di CO₂ è stato interrotto nei periodi di buio. Questi esperimenti hanno dimostrato che in condizioni limitanti di anidride carbonica, e in assenza di luce, la capacità delle microalghe di consumare i composti organici viene ripristinata e la presenza del substrato aumenta la quantità di biomassa prodotta.

La velocità di crescita microalgale è influenzata, inoltre, dalla disponibilità della luce che, se poco intensa, può essere limitante, mentre, se presente in eccesso, può portare a fenomeni di fotosaturazione o fotoinibizione, con una conseguente perdita in produttività (Carvalho et al., 2011; Cuaresma et al., 2011; Brindley et al., 2011). Allo scopo di valutare l'effetto delle intensità luminose, gli esperimenti sono stati condotti in reattori a pannello sottile, appositamente progettati e costruiti. In tale sistema sono state effettuate curve di crescita a diverse intensità luminose, con particolare attenzione anche ai cicli luce-buio ad alta frequenza, che potrebbero evitare i fenomeni di fotoinibizione alle elevate intensità.

I risultati mostrano che la velocità di crescita di *N. salina* aumenta linearmente all'aumento dell'intensità luminosa fino a valori di 150 $\mu\text{E m}^{-2} \text{s}^{-1}$. In questo intervallo, quindi, la luce è da considerarsi limitante per la crescita. Oltre tali valori, invece, la velocità si assesta e le cellule mostrano segnali visibili di stress, con ingiallimento delle colture per l'accumulo di pigmenti fotoprotettori, e l'aumento del contenuto di lipidi, anche quando nel terreno è presente un elevato contenuto di azoto. Se sottoposte a cicli ad alta frequenza luce buio, ad elevate intensità, le cellule mostrano una diminuzione dei segnali di stress, suggerendo la possibilità di evitare tali fenomeni di fotosaturazione e fotoinibizione mediante mescolamento in un ipotetico fotobioreattore, che esponga ciclicamente le cellule alla superficie di esposizione, grazie a cicli di mescolamento (Zijffers et al., 2010). I risultati hanno dimostrato che la frequenza è cruciale nell'evitare fenomeni di stress, perché all'aumento dei tempi di esposizione aumentano i danni agli apparati fotosintetici, con una conseguente drastica diminuzione della produttività. Il tempo di *flash light* che ottimizza l'assorbimento della luce sembra essere nell'ordine dei 10 ms. Queste indicazioni devono essere prese in considerazione nella progettazione di un fotobioreattore, con particolare riguardo alle frequenze di mixing, che possono aumentare le performance di crescita esponendo alla superficie di esposizione le cellule a cicli tali da ridurre i fenomeni di foto inibizione.

In questa tesi di dottorato, inoltre, sono stati effettuati degli esperimenti di *screening* di una specie algale d'acqua dolce, con la quale sono stati inoltre effettuati degli esperimenti volti a verificare la capacità di utilizzare fumi di combustione e azoto di acque di processo di

stabilimenti industriali. Questa parte del progetto ha visto, quindi, l'identificazione di un ceppo di microalghe di acqua dolce che potesse essere usato per la produzione di olio in un impianto che utilizzi tali sottoprodotto.

Dai risultati ottenuti per le specie e, assumendo rese tipiche di fotobioreattori industriali esistenti, per *N. oleabundans* i dati fanno ipotizzare una produttività annuale possibile di olio di circa 25 t/ha (e un limite teorico di circa 130 t/ha); mentre per *B. braunii* i dati sperimentali indicano una produttività possibile di circa 35 t/ha (limite teorico: circa 170 t/ha). Si è, inoltre, verificato sperimentalmente, che l'acqua di processo e i fumi disponibili da un reale stabilimento industriale consentono la crescita di *N. oleabundans*, pur con velocità inferiore).

L'attività sperimentale di questo progetto di dottorato ha visto, inoltre, il design e la costruzione di un fotobioreattore a scala laboratorio per la produzione in continuo di biomassa. In tale reattore, configurato a pannello, è stata ottenuta la produzione di *N. salina* per una durata di 100 giorni complessivi, con una produttività costante di biomassa alla concentrazione di circa 1 g/L DW. Essendo in condizioni di azoto non limitante, la biomassa prodotta ha mantenuto un basso contenuto di lipidi. Come detto in precedenza, quindi, per questa specie è necessario un secondo step per l'accumulo di lipidi.

L'ultima parte di questa tesi è stata dedicata a calcoli di produttività teorica di biomassa algale, mediante simulazioni con il software Aspen Plus™.

Per simulare un fotobioreattore per microalghe con il software Aspen Plus, è stato necessario impostare un componente non convenzionale (la biomassa), e una *subroutine* in Fortran per impostare la cinetica e la stechiometria di reazione nel blocco reattore. In tal modo è stato possibile simulare il processo di produzione di biomassa algale. Dai risultati della simulazione è stato possibile, inoltre, fare delle considerazioni sulla geometria del reattore, e in particolare sulla profondità dello stesso, in grado di garantire elevati valori di produttività. E' sempre necessario, comunque, tener conto dei vincoli termodinamici di produttività, che sono imposti dalla radiazione solare incidente. Le analisi di sensitività sul fotobioreattore hanno mostrato che, a parità di produttività, l'aumento della concentrazione di biomassa in ingresso comporta un volume di reazione minore, e tempi di permanenza minori.

In sintesi, la produzione di olio vegetale da microalghe, sebbene presenti ancora alcuni aspetti che devono essere approfonditi, soprattutto per quanto riguarda le conoscenze fisiologiche e biologiche di tali organismi, sembra essere promettente. La tecnologia, infatti, pur agli albori, potrebbe dare un contributo determinante all'approvvigionamento di biocarburanti, in modo ecocompatibile ed energeticamente sostenibile.

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ABSTRACT

This PhD research project was focused on microalgal oil production for biofuel. The work includes mostly an experimental part on microalgal species selection and optimization of growth conditions and a part of process simulation and photobioreactor design.

After an overview of literature on the algal biology and cultivation and photobioreactor design, the experimental activities started with the set up of materials, methods and experimental apparatus, several microalgal species were screened, in order to select the most promising ones from an industrial point of view. In addition, an experimental apparatus was set up for optimizing growth conditions, under non-limiting CO₂ supply. The effect of light and of other relevant operating variables on growth were addressed and discussed, and some suggestions to better understand the process behavior were given with respect to lipid content maximization, carbon dioxide and nitrogen supply, and illumination conditions. The possibility of exploiting mixotrophy to support algal growth overnight or in dark zones of a photobioreactor was also investigated. Finally, a continuous photobioreactor was designed and built, in order to test the feasibility of the algal biomass production in an industrial continuous process operated at steady-state.

Together with experiments, a process simulation work was also carried out, by using the software Aspen Plus™. This part of the thesis was focused on the calculation of the microalgal biomass production and photobioreactor performances under different operating conditions.

INTRODUCTION

Today about 85% of global energy demand is produced from fossil fuels. In recent decades, the world has been confronted with an energy crisis, associated with irreversible depletion of traditional sources of fossil fuels. The reduction of crude oil reserves and difficulties in their extraction and processing is increasing its cost. According to the International Energy Agency World Energy Outlook, the demand for energy will probably increase by 40% between now and 2030 (Malcata et al., 2011), as depicted in fig.1.

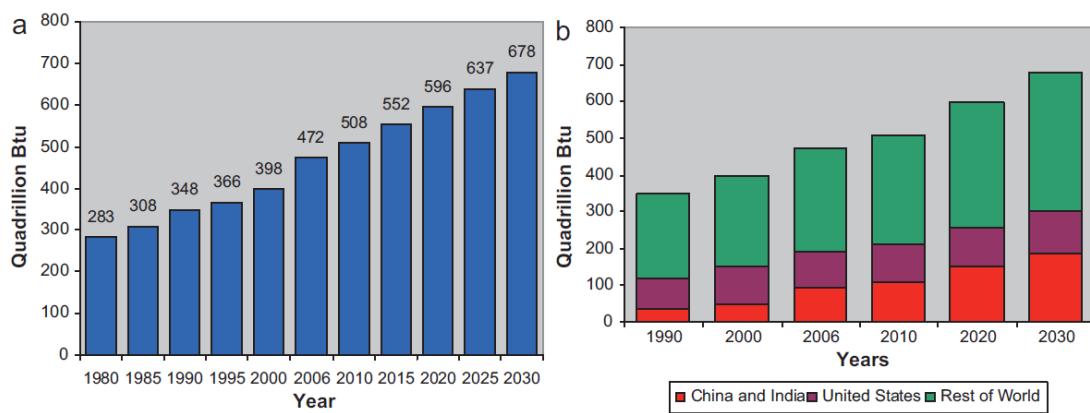


Figure 1. (a) World marketed energy consumption. (b) Marketed energy use by region (Ahmad et al., 2011).

In addition, extensive utilization of fossil fuels has led to global climate change, environmental pollution, and health problems (Mata et al., 2010). Many countries are thus turning their attention to the development of new, clean, and sustainable energy sources. For example, in many regions around the world targets for CO₂ reduction have been established, in order to meet the sustainability goals agreed under the Kyoto Protocol.

Presently, many options are being studied and implemented in practice, with different degrees of success, and in different phases of study and implementation. Examples include biofuels, solar energy (thermal and photovoltaics), hydroelectric, geothermal, wind, and carbon

sequestration, among others (Jacobson, 2009). Each one has its own advantages and problems and, depending on the area of application, different options will be better suited.

A major issue is related to the transportation sector, where there are currently no relevant alternatives to fossil fuels. In addition, one important goal is to take measures for transportation emissions reduction, such as the gradual replacement of fossil fuels by renewable energy sources.

Among the various potential sources of renewable energy, biofuels are promising and are expected to play a crucial role in the global energy infrastructure of our future (Chen and Walker, 2011), in particular for supporting the energy demand for transportation. The most common biofuels are biodiesel and bio-ethanol, which can replace diesel and gasoline, respectively, in today cars with little or none modifications of vehicle engines. Thus, they can be produced using existing technologies and be distributed through the available distribution system. For these reasons, biofuels are currently pursued as a fuel alternative that can be easily applied until other options harder to implement, such as hydrogen, may be available (Mata et al., 2010). Although biofuels are still more expensive than fossil fuels their production is increasing all around the world.

Among the various potential feedstocks, the more interesting one seems to be microalgae (Chisti, 2007; Shi et al., 2011; Mata et al., 2010; Scott et al., 2010). In fact, biodiesel from microalgae is currently being evaluated to determine if it is a viable renewable source to replace oil. In 1970, the Aquatic Species Program grew out a national effort to produce biodiesel from high lipid content algae. In 1996, the biofuels program shifted its focus to corn and soybean technologies to produce bioethanol, due to the fact that corn and soybean fuels were considered a proven technology. However, it is clear that these sources cannot satisfy the entire energy demand. Comparing different crops used for biodiesel and their respective oil yields, each source takes up large areas of land leading to significant ecological impact (Csavina et al., 2011).

Consequently, a renewed interest in microalgae is now growing, as a source that might have the potential to supply substantial amounts of energy.

Microalgae clearly offer several advantages if compared with biomass resources used for first- and second generation biofuels, as recently reviewed by Amaro et al. (2011) and Takeshita (2011):

- They grow rapidly and can double their biomass as much as many times a day during exponential growth (Chisti, 2007).
- They have significantly high oil and biomass yield per unit area: yield of oil from microalgae could be 7–31 times greater than the next best crop, palm oil (Schenk et al., 2008). Finding high lipid producing strains (Griffiths and Harrison, 2009; Rodolfi et al., 2009; Mutanda et

al., 2011) and selecting the appropriate culturing and processing conditions are critical to realize the potential and large scale adoption of advanced algal biofuels (Rodolfi et al., 2009; Brennan and Owende, 2010; Greenwell et al., 2010; Huang et al., 2010).

- They showed also an efficient use of nutrients due to the aqueous environment and their recycling, requiring only sunlight and a few simple and non-expensive nutrients (in particular nitrogen and phosphorus), including those associated with local, specific environments. In addition, they have the ability to be cultivated in salt water or wastewater ponds on marginal land, with reduced competition with food production and other products derived from crops. In spite of their growth in aqueous media, microalgae need rates of water renewal lower than terrestrial crops need as irrigation water, so the load on freshwater sources is strongly reduced: the amount of water needed to produce an energy unit being estimated to be 74% smaller for microalgal biodiesel production than for rapeseed biodiesel production (Yang et al., 2011).
- The biomass resulting after oil extraction can be processed into ethanol or natural gas and could provide other types of renewable biofuels. These include methane produced by anaerobic digestion of the algal biomass, biodiesel derived from microalgal oil, and photobiologically produced biohydrogen (Chisti, 2007) (see fig 1). The extracted biomass could also simply used as organic fertilizer, owing to its high N:P ratio; or merely burned for energy cogeneration (electricity and heat). Alternatively, a wide range of fine chemicals and bulk products – e.g. polyunsaturated fatty acids, natural dyes and antioxidants, may also be extracted from the spent biomass, depending on the species at stake (Demirbas, 2011).
- In summary, microalgae are efficient producers of natural oils, sequester carbon dioxide thereby reducing greenhouse gases (Ho et al., 2011), and do not compete with food production or deplete soil nutrients. Concerning CO₂, 1 kg of dry algal biomass requires about 1.83 kg of CO₂, which can readily be obtained from industrial flue gases via bio-fixation (Amaro et al., 2011).

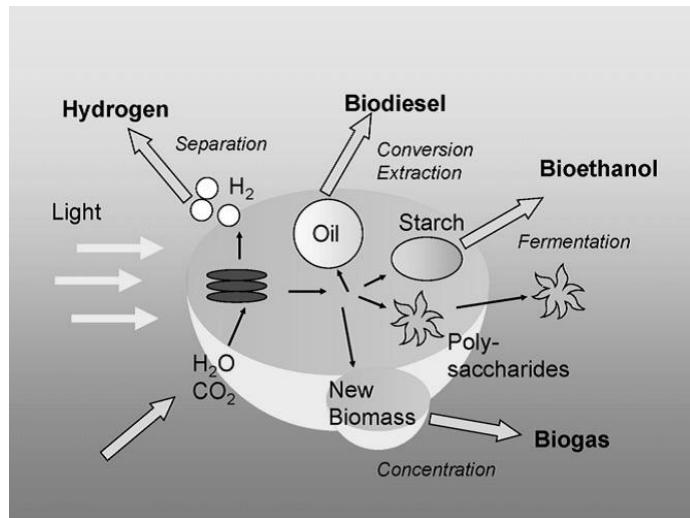


Figure 2. Potential pathways from microalgae to fuels (Posten and Schaub, 2009).

Microalgae indeed have some disadvantages. First, their production requires substantial infrastructure and incurs large capital costs: biomass production cost per GJ of biodiesel in 2008 was estimated to be 14.5 times higher for microalgal biodiesel than for rapeseed biodiesel (Kovacevic and Wesseler, 2010).

Furthermore, biomass concentration in the microalgal culture is low, which in combination with the small size of microalgal cells makes the harvest of microalgal biomass relatively costly, and drying harvested microalgal biomass is an energy-consuming process due to its large water content.

However, because these problems are expected to be overcome or minimized by technology development and because of the distinctive features of microalgae, microalgae are receiving increasing attention, particularly as a high-potential source for biodiesel production (Takeshita, 2011)

This thesis reports a starting project focused on microalgae cultivation for oil production. The topics of the thesis are organized and subdivided in chapters as follows.

Chapter 1 is an introductory discussion on microalgae and oil production with the aim of describing what is the world situation with regard to algal cultivation. Particular attention is devoted to the description of current most interesting species used in this field, optimization of growth conditions and a brief review of culture systems and harvesting techniques.

In **Chapter 2** materials and methods used and optimized in the following are reported. In addition, a screening of algal species is presented.

Chapters 3 and 4 report the experimental work carried out about autotrophic and mixotrophic cultivation of selected species.

The aim of chapter 5 is to select a freshwater species that could be used in a photobioreactor fed with by-products from an industrial process, that contain high amounts of CO₂ and nitrogen.

Chapter 6 summarizes results about the effect of continuous and pulsed light on microalgae growth and photosynthetic efficiency in a flat plate culture system.

Chapter 7 describes experiments about the continuous production of microalgal biomass in a flat plate reactor operated at steady-state.

Finally, in **chapter 8**, the entire process of algal biomass production is described, and simulated with the process simulator Aspen Plus™, to show the relevant operating parameters in massive algal production by a photobioreactor.

This research work was done at the Department of Chemical Engineering Principles and Practice “I. Sorgato” (DIPIC) of the University of Padova, under the supervision of Prof. Alberto Bertucco.

I would like to thank also Prof. Giorgio Giacometti and Dr Tomas Morosinotto (Department of Biology, Padova) for a very worthwhile collaboration in research activities.

As a tangible result of the work completed during the three years spent for my PhD school, a number of publications and presentations to conferences have been produced, as listed below. In addition, five research activities as master thesis and three as bachelor thesis were co-tutored.

Papers in Refereed Journals

1. Simionato, D., Sforza, E., Corteggiani Carpinelli, E., Bertucco A., Giacometti, G.M., Morosinotto, T., 2011. Acclimation of *Nannochloropsisgaditana* to different illumination regimes: Effects on lipids accumulation. *Bioresour. Technol.* 102 6026–6032
2. Sforza, E., Cipriani, R., Morosinotto., T., Bertucco, A., Giacometti, G.M., 2011. Excess CO₂ supply inhibits mixotrophic growth of *Chlorella protothecoides* and *Nannochloropsisissalina*. *Bioresour. Technol.*, doi:10.1016/j.biortech.2011.10.025
3. Sforza, E., Bertucco, A., Morosinotto., T., Giacometti, G.M., 2011. Photobioreactors for microalgal growth and oil production with *Nannochloropsisissalina*: from lab-scale experiments to large-scale design. *Chemical Engineering Research and Design (ChERD)*, *in press*.

Papers submitted to Refereed journals

1. Sforza, E., Simionato, D., Bertucco A., Giacometti, G.M., Morosinotto, T. Optimized light/dark cycles in photobioreactors has a huge impact on photosynthetic productivity. Submitted to Plos One.

Papers in preparation

1. Identification and characterization of a novel lipid-hyperproducer strain of *Scenedesmus* sp
2. Production of *N.salina* biomass in a continuous photobioreactor: productivity, mass balance and photosynthetic efficiency

Others papers

1. Sforza E., Bertucco A., Morosinotto T. and Giacometti G.M., 2010. Carburanti da microalge: ottimizzazione dei parametri di crescita. ICP 10, 90-95.
2. Sforza E., Bertucco A., Giacometti G.M. Biocarburanti e biomasse: le microalge sono la risposta? Ottimizzazione delle condizioni di coltura di microalge nell'ottica di una produzione industriale. Atti e Memorie dell'Accademia Galileiana di Scienze Lettere ed Arti, 2011.

Papers or abstracts in Conference Proceedings

1. Sforza E., Bertucco A., Morosinotto T., Giacometti G.M. Vegetal oil from microalgae: species selection and optimization of growth parameters. Presented at "Biological processes as a possible source for renewable Energy" Conference, November 02- 03, 2009, Roma, Italy.
2. Giacometti G.M., Morosinotto T., Sforza E., Bertucco A. Water Bioremediation by MicroalgaeCultivation. Presented at Italian-Israeli Scientific Seminar "THE VIRTUOUS CYCLE FROM DEPURATION TO CLEAN ENERGY" November 18 , 2009, Tel Aviv, Israel
3. Sforza E., Bertucco A., Morosinotto T., Giacometti G.M. Vegetal oil from microalgae: from laboratory measurement to reactor design. EMCC5 Conference, March 2010, Antalya.
4. Sforza E., Bertucco A., Morosinotto T., Giacometti G.M. Vegetal oil from microalgae: species selection and optimization of growth parameters. IBIC 2010 Conference, April 11- 14, 2010, Padova Italy.
5. Sforza E., Bertucco A., Morosinotto T., Giacometti G.M. Vegetal oil from microalgae: mixotrophic growth in view to large-scale production. IBS 2010 Conference, September 14- 18, 2010, Rimini, Italy.
6. Simionato D., Sforza E., Corteggiani-Carpinelli E., Segalla A., Pistorello C., Bertucco A., Giacometti G.M., Morosinotto T. Acclimation of *Nannochloropsis gaditana* to different illumination regimes: effects on lipids accumulation. BIO PhD Day March 31th-April 1st 2011, Complesso Biologico "A. Vallisneri", Università di Padova.
7. Sforza E., Bertucco A., Giacometti G.M. Biocarburanti e biomasse: le microalge sono la risposta? Ottimizzazione delle condizioni di coltura di microalge nell'ottica di una produzione industriale. Accademia Galileiana, May, 20, 2011, Padova, Italy.
8. Sforza E., Bertucco A., Bezzo F., Morosinotto T., Giacometti G.M. Effect of light intensity and high frequency pulsation on autotrophic growth of *Nannochloropsis salina* in flat plate reactor. 1st International Conference Algal BBB, July, 17-20, 2011. St. Louis, USA.
9. Sforza E., Cipriani R., Bertucco A., Morosinotto T., Giacometti G.M. Mixotrophic growth of *Chlorella protothecoides* and *Nannochloropsis salina* in view of sustainable

- photobioreactor development. 1st International Conference Algal BBB, July, 17-20, 2011. St. Louis, USA.
- 10.Simonato D., Sforza E., Corteggiani Carpinelli E., Bertucco A., Giacometti G.M., Morosinotto T. Investigating the connection between photosynthetic efficiency and lipids productivity in *Nannochloropsis* cultures. 1st International Conference Algal BBB, July, 17-20, 2011. St. Louis, USA.
- 11.Sforza E., Morosinotto T., Bezzo F., Giacometti G.M., Bertucco A. Autotrophic production of *Nannochloropsis salina* in a continuous photobioreactor. ISAF 2011, October, 10-14, 2011. Verona, Italy.
- 12.Ramos Tercero E.A., Sforza E., Palma G., Giacometti G.M., Bertucco A. Evaluation of energy efficiency and recovery in microalgal production processes. International Algae Congress, December, 1-2, 2011. Berlin.

Participation to Scientific Conferences

1. Sforza E., Giacometti G., Bertucco A. Impiego di microalghe per la produzione di biocarburanti. 3° Workshop del Programma T.A.C.E.C. “Fonti rinnovabili: il contributo della chimica alla sostenibilità” Federchimica, June, 16, 2009, Milano, Italy. ORAL PRESENTATION
2. Sforza E., Bertucco A., Morosinotto T., Giacometti G.M. Vegetal oil from microalgae: mixotrophic growth in view to large-scale production. IBS 2010 Conference, September 14- 18, 2010, Rimini, Italy. ORAL PRESENTATION
3. Sforza E., Bertucco A., Giacometti G.M. Biocarburanti e biomasse: le microalghe sono la risposta? Ottimizzazione delle condizioni di coltura di microalghe nell’ottica di una produzione industriale. Accademia Galileiana di Scienze Lettere ed Arti, May, 20, 2011, Padova, Italy. ORAL PRESENTATION
4. Bertucco A., Sforza E. Quali fonti rinnovabili in Italia?. Conferenza “Lo sfruttamento dell’energia solare: prospettive in Italia dopo il 12 giugno”, June, 24, 2011, Padova, Italy. ORAL PRESENTATION
5. Sforza E., Bertucco A., Bezzo F., Morosinotto T., Giacometti G.M. Effect of light intensity and high frequency pulsation on autotrophic growth of *Nannochloropsis salina* in flat plate reactor. 1st International Conference Algal BBB, July, 17-20, 2011. St. Louis, USA. POSTER
6. Sforza E., Cipriani R., Bertucco A., Morosinotto T., Giacometti G.M. Mixotrophic growth of *Chlorella protothecoides* and *Nannochloropsis salina* in view of sustainable photobioreactor development. 1st International Conference Algal BBB, July, 17-20, 2011. St. Louis, USA. POSTER
7. Sforza E., Morosinotto T., Bezzo F., Giacometti G.M., Bertucco A. Autotrophic production of *Nannochloropsis salina* in a continuous photobioreactor. ISAF 2011, October, 10-14, 2011. Verona, Italy. ORAL PRESENTATION
8. Sforza E., Ramos Tercero E.A., Palma G., Giacometti G.M., Bertucco A. Evaluation of energy efficiency and recovery in microalgal production processes. International Algae Congress, December, 1-2, 2011. Berlin. POSTER

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2. 10/07/2009, Life Long Learning Intensive course “Supercritical Fluids- Green solvent in Chemical Engineering”, KOC University, Istanbul, Turkey, **2CFU**.
3. 29/07/2010, “Fenomeni di trasporto I”, tenuto dalla Prof.ssa Giomo, II semestre anno accademico 2008-2009, Corso di Laurea Ingegneria Chimica, Università di Padova, **6CFU**;
4. 15/11/2010-“Simulazione fluidodinamica e di processo”, tenuto dall’Ing. Bezzo, I semestre anno accademico 2009-2010. Università di Padova, **6CFU**.
5. 08/11/2010, “Processi e impianti industriali chimici 2”, tenuto dal Prof. Guarise, II semestre anno accademico 2008-2009, Corso di Laurea in Chimica Industriale, Università di Padova, **6CFU**.
6. 22/04/2009- 12/05/2009, Corso base per i dottorandi di “Analisi del rischio”, tenuto dal Prof. Maschio. Università di Padova.
7. 6-11/06/2009, Scuola Nazionale Gricu per Dottorandi “Termodynami ca – Energia”, Muravera, Cagliari;
8. 12-18/09/2010, Scuola Nazionale Gricu per Dottorandi “Reattoristica Chimica – Applicazioni Biomediche”, Rimini;
9. 21-24/09/2010, Corso specialistico per dottorandi: “Towards new reactors”, tenuto dal Prof. Tapio Salmi , AboAkademi di Turku (Finlandia);
10. 25/09/2011-1/10/2011, Scuola Nazionale Gricu per Dottorandi “Mathematical Methods for Chemical Engineering – Nanotechnologies”, Santa Margherita di Pula, Cagliari;
11. Cicli di seminari per dottorandi, DIPIC, Università di Padova;

Didactis

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2. Tutor Junior per il corso di: “Fondamenti dell’Ingegneria di Processo”, prof. Alberto Bertucco. A.A. 2010-2011 (primo semestre dell’anno accademico)

Thesis cotutored

Bachelor thesis:

1. Title: PRODUZIONE DI BIOMASSA ALGALE: ANALISI E SIMULAZIONE DI FOTOBIOREATTORE. Corso di laurea: Ingegneria Chimica. Student: Alessandra Tosato. Anno Accademico 2009-2010
2. Title: IL RUOLO DELL’AZOTO NELLA PRODUZIONE DI LIPIDI DA COLTURE MICROALGALI. Studente: Pascal Albanese. Corso di laurea: Biologia. Anno Accademico 2009-2010
3. Title: INFLUENZA DELL’ILLUMINAZIONE SULLA CRESCITA DI *Nannochloropsis salina* PER LA PRODUZIONE DI OLIO A SCOPO ENERGETICO. Student: Samson Akanbi. Corso di laurea: Ingegneria Chimica. Anno Accademico 2010-2011

Master thesis:

1. Title: EFFETTO DELL'INTENSITÀ DI LUCE SULLA CRESCITA DI MICROALGHE: ANALISI SPERIMENTALE E IDENTIFICAZIONE DI MODELLI PREDITTIVI. Corso di laurea: Ingegneria Chimica. Student: Katia Adami. Anno accademico 2010-2011
2. Title: PRODUZIONE DI MICROALGHE IN FOTOBIOREATTORI: INFLUENZA DELLA LUCE E SFRUTTAMENTO DELLA BIOMASSA ESAUSTA. Corso di laurea: Ingegneria Chimica. Student: Gianmarco Palma. Anno accademico 2010-2011
3. Title: EFFETTO DELL'INTENSITÀ DI LUCE SULLA CRESCITA E ACCUMULO DI LIPIDI IN *Scenedesmus obliquus* IN REATTORI IN CONTINUO. Studente: Barbara Gris. Corso di laurea: Biotecnologie Industriali. IN CORSO
4. Title: DESIGN DI UN FOTOBIOREATTORE PER LA COLTIVAZIONE DI *Scenedesmus obliquus* E LA PRODUZIONE DI OLI COMBUSTIBILI. Studente: Mattia Enzo. Corso di laurea: Ingegneria Chimica. IN CORSO

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CHAPTER 1

OIL FROM MICROALGAE: STATE OF THE ART

This chapter addresses the topic of the thesis and reviews the literature on algal biology and cultivation and photobioreactor design. In addition, an overview of alternate nutrient sources for cultivation are reported. This chapter also reports advances achieved in the last years and the future trends in research and from an industrial perspective.

1.1. Oil from microalgae: a process outlook

Producing oil from microalgal biomass might provide significant benefits for alternate fuel supply (Chisti, 2007). A conceptual process to produce microalgal oil for biodiesel is shown in figure 1.1.

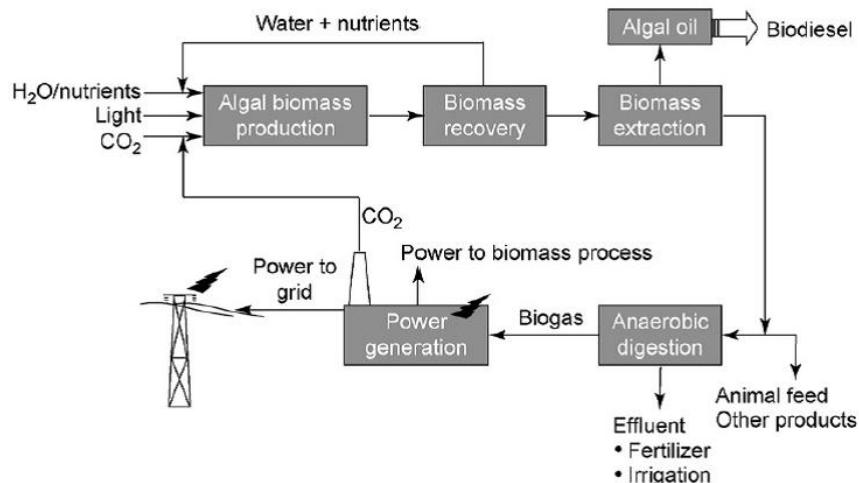


Figure 1.1 Theoretical block flow diagram of oil production process from microalgae (Chisti, 2008).

In this scheme, water, inorganic nutrients, carbon dioxide and light are provided to the microalgal culture during the biomass-production stage. In the biomass recovery stage, the cells suspended in the broth are separated from water and residual nutrients, which are then recycled to the biomass production stage. The recovered biomass is used for extracting the algal oil that is further converted to biodiesel in a separate process. Some of the spent

biomass can be used as animal feed and for recovering other possible high value products that might be present. Most of the biomass undergoes anaerobic digestion, which produces biogas to generate electricity. Effluents from the anaerobic digester could be used as a nutrient-rich fertilizer and as irrigation water. Most of the power generated from the biogas produced is consumed within process and any excess energy is sold to grid. Carbon dioxide emissions from the power generation stage are fed into the biomass photobioreactor (Chisti, 2008).

Currently, some issues concerning algal biofuel production are still unsolved, and considerable amount of R&D are underway: industrial tests are ongoing in order to improve system efficiency and cost-competitiveness with traditional fuel industries. Thus, the algal biofuel production spectrum is currently comprising a fairly complex set of steps that begins with upstream algal strain selection and concludes with the conversion of algal biomass into a finished energy product. The state of the technology is investigated with respect to key steps: algal strain selection, algal cultivation, harvesting and dewatering, oil/biomass separation and end-use fuel production (see figure 1.2).

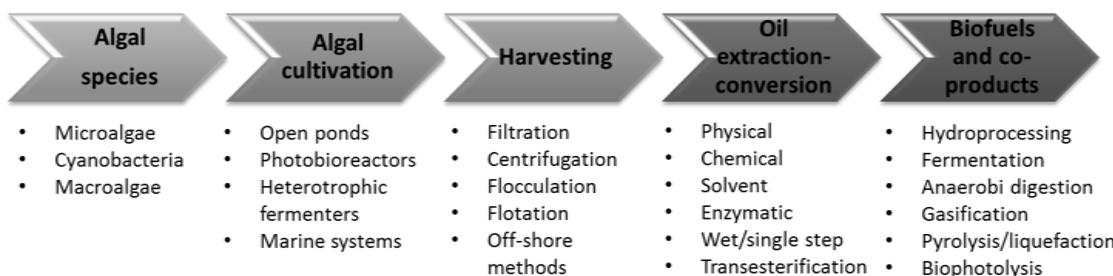


Fig.1.2 Research issues (modified from IEA Energy Technology Network Report 2011)

Harvesting and dewatering are often collectively considered the greatest bottleneck to scaling up algal biofuel production. A large percent of the energy and cost is consumed in order to obtain sludge dry enough to carry out lipid extraction and/or fuel conversion. The main techniques presently applied in the harvesting of microalgae include centrifugation, flocculation, filtration and screening, gravity sedimentation, flotation, and electrophoresis techniques (Gong and Jiang, 2011; Uduman et al., 2010). The cost of algae harvesting can be high, since the mass fractions in culture broth are generally low. The selection of harvesting technique is dependent on the properties of microalgae (Danquah et al., 2009), such as density, size, the value of the desired products (Brennan and Owende, 2010). The cost of harvesting strongly affects the cost of final product, as reported by several LCA studies (Stephenson et al., 2010; Clarens et al., 2010): it has been claimed that solvent extraction of oil from microalgae lead to a significant energy penalty, especially if biomass is fully dried prior to extraction. 1 MJ of energy obtained from the final biodiesel would require a global input of

1.66 MJ of energy, with a further 1.23 MJ being potentially recovered from the microalgal waste left after oil extraction (Singh and Gu, 2010). Thus, the international research is focused on the extraction of oil directly from the suspension, after a step of cell lysis (<http://www.originoil.com>; Xu et al., 2011; Halim et al., 2011). The most promising technique seems the one using acoustic and electromagnetic fields, that could make more feasible the whole process from an economic point of view (Cooney et al., 2009).

However, although the downstream process are considered the bottleneck as far as the process feasibility is concerned, it must to be considered that the production step is still far to be well known. In fact, both the selection of the species for the specific application is crucial and the system cultivation must be designed for the specific strain selected.

Thus, the aim of this thesis is to study the biomass production step, in order to better understand the feasibility of microalgal production at the industrial scale. In particular, in this work, we focused on the selection of biomass species for specific purposes and on the definition of a suitable photobioreactor design.

1.2. Microalgae: selection of the species

1.2.1. Microalgae definition

Phycologists regard as an alga any organisms with chlorophyll A and a thallus not differentiated into roots, stem and leaves (Lee, 1989). Cyanobacteria are included in this definition, even though they are prokaryotic organisms. Microalgae are present all over the world. They are mainly distributed in waters, but are also found on the surface of all types of soils. Although they are generally free-living, a certain number of microalgae live in symbiotic association with a variety of other organisms (Tomaselli, 2003; 2007).

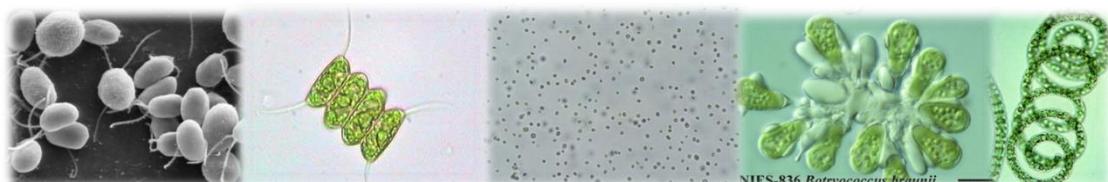


Figure 1.3. Microalgal species: *Chlamydomonas reinhardtii*, *Scenedesmus quadricauda*, *Nannochloropsis salina*, *Botryococcus braunii*, *Arthrospira platensis* respectively.

1.2.2. Morphology, physiology and systematics

Microalgae may have different types of cell organization: unicellular, colonial and filamentous (fig.1.3). Some unicellular microalgae are nonmotile, but swimming motility may occur, essentially due to the presence of flagella.

The microalgal cell wall is generally composed by a microfibrillar layer of cellulose, which may be surrounded by an amorphous layer. The cell wall may be silicified or calcified, and it may be

strengthened with plates and scales. Outside the outer amorphous layer a laminated polysaccharide investment may occur.

The cytoplasm contains the nucleus and different kinds of organelles and compartments formed by invagination of the plasma membrane and endoplasmic reticulum. Among the organelles there are: chloroplast, Golgi apparatus, endoplasmic reticulum, ribosomes, mitochondria, vacuoles, contractile vacuoles, plastids, lipid globules, flagella, and microtubules. The nucleus is bounded by a double nuclear membrane; it contains the nucleolus and DNA distributed among the chromosomes, and undergoes division by mitosis.

Vegetative reproduction by cell division is widespread for algae and is related, in many species, to an increase in cell or colony size. Although sexual reproduction occurs in the life-history of most of the species, it is not a universal feature in algae.

Algae have been traditionally classified according to their pigment content and this characteristic continues to be of a certain importance. The current systems of classification of algae are based on the following main criteria: kinds of pigments, chemical nature of storage products and cell wall composition. Additional criteria take into consideration the occurrence and structure of flagellate cells, scheme and path of nuclear and cell division, presence of an envelope of endoplasmic reticulum around the chloroplast, and possible connection between the endoplasmic reticulum and the nuclear membrane. Lee (1989) separated the algal divisions into four groups. The first one includes prokaryotic algae: Cyanobacteria and Prochlorophyta. The other groups are classified with respect to the evolution of the chloroplast, and include the eukaryotic algae. The second one, which includes Glaucophyta, Rhodophyta and Chlorophyta, has the chloroplast surrounded by two chloroplast membranes only. The third and fourth group have the chloroplast surrounded respectively by one (Dinophyta and Euglenophyta) or two additional membranes of the endoplasmic reticulum (Cryptophyta, Chrysophyta, Prymnesiophyta, Bacillariophyta, Xanthophyta, Eustigmatophyta, Raphidophyta and Phaeophyta). The phylum Prochlorophyta contains chlorophylls a and b. The systematic position of the various algal groups has changed many times over the years (Tomaselli, 2003; 2007).

1.2.3. Photosynthesis

Microalgae, including cyanobacteria, have the capacity to absorb light energy, i.e. photons, and to store it as chemical energy via the formation of chemical bonds. Oxygenic photosynthesis can be expressed as a redox reaction driven by light energy, which is harvested by chlorophyll molecules, where carbon dioxide and water are converted to carbohydrates and oxygen. The conversion is traditionally divided in two stages, the so-called “light reactions” and “dark reactions” (fig 1.4). The light dependent reactions involve the capture of photons and their conversion to energy currency as NADPH and ATP. These

reactions are absorption and transfer of photon energy, trapping of this energy, and generation of a chemical potential. The latter reaction follows two routes: a first one, which generates NADPH due to the falling of the high energy excited electron along an electron transport system and a second one that produces ATP by means of a proton gradient across the thylakoid membrane. Water splitting is the source of both electrons and protons. Oxygen is released as a by-product of the water splitting. The reactions of the second group are the “light-independent reactions,” and involve the sequence of reactions by which this chemical potential is used to fix and reduce inorganic carbon (i.e. CO_2) in triose phosphates (Masojídek et al., 2007).

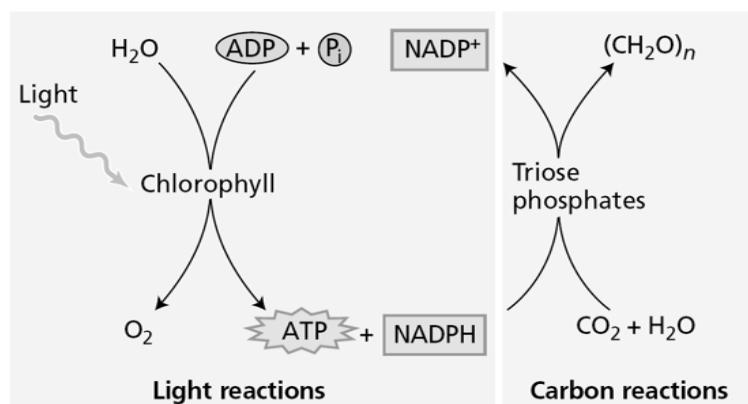


Fig. 1.4. Photosynthetic pathway: light reaction for energy capture and dark reaction to fix carbon (Masojídek, Kobli?ek and Torzillo, 2003; 2007).

In producing microalgal biomass, energy efficiencies for converting solar energy into chemical energy can be achieved which are higher in comparison to terrestrial plants. Based on present understanding, it can be expected that microalgae can convert up to 5% of the incoming sunlight energy to biomass (PCE = photoconversion efficiency) (Schenk et al., 2008). This high yield together with the different growing conditions gave reason for some authors to call microalgae the “3rd generation biomass”.

1.2.4. Microalgal lipid content and productivity –species selection

The first large-scale collection and screening of oleaginous algae dates back to 1978, when the Aquatic Species Program (ASP) was launched by the U.S. National Renewable Energy Laboratory (NREL), for the production of biodiesel from high lipid-content algae. With 8 years of effort, over 3,000 strains were collected and eventually around 300 species were identified as oil-rich algae (Sheehan et al. 1998).

When algal cells are actively growing, polar membrane lipids (phospholipids and glycolipids) generally predominate, but as the cell enters stationary-phase many species accumulate triacylglycerols (TAGs) (Hu et al., 2008). Microalgal species can be induced to accumulate

substantial contents of lipids also by changing environmental conditions. Although average lipid contents vary between 1% and 70%, some species may reach 90% (w/w DW) (Sheehan 1998, Chisti, 2007, Li et al., 2008). Nitrogen levels, light intensity, temperature, salinity, CO₂ concentration and harvesting procedures have all been demonstrated to influence the lipid content of microalgae (Brennan and Owende, 2010). Nitrogen deficiency, in particular, is often a trigger to induce the switch to lipid accumulation. In addition, the effects of light intensity on the composition of lipids produced has been extensively studied and, in general, low light intensities result in mainly membrane polar lipids, whereas high intensities result in an increase of triacylglycerols (TAGs) storage (Sukenik and Carmeli, 1990).

Several differences are apparent in table 1.1 among the various species listed, and even within the same genus. Many algae produce significant levels of lipids, with lipid contents as high as 75% for *Botryococcus braunii*, but the total lipid yield depends on both areal productivity and lipid content (Mata et al., 2010). In addition, some species might be not considered because of their low growth rates or inability to produce highly concentrated cultures. For instance, the high oil content *Botryococcus braunii* is associated with a low productivity (Metzger and Largeau, 2005). Thus, lipid content is not the only factor determining the oil-producing ability of microalgae. Instead, both lipid content and biomass production need to be considered simultaneously. Hence, lipid productivity, representing the combined effects of oil content and biomass production, is a more suitable performance index to indicate the ability of a microalga with regard to oil production. The highest lipid productivity reported in the literature is about 179 mg/L d by *Chlorella* sp. under phototrophic cultivation using 2% CO₂ with 0.25 v/v of aeration (Chiu et al., 2008).

The majority of microalgae routinely used in research laboratories are model strains and in most cases they were chosen for their ease of cultivation and not necessarily for their lipid production, or their potential as a biofuels precursor. The most common microalgae (viz. *Chlorella*, *Dunaliella*, *Isochrysis*, *Nannochloris*, *Nannochloropsis*, *Neochloris*, *Nitzschia*, *Phaeodactylum* and *Porphyridium* spp.) possess oil levels between 20% and 50%, along with interesting productivities.

Besides high productivities, selection of the most adequate species for biodiesel production should take other factors into account, e.g. the ability to uptake available nutrients or grow under specific environmental conditions and the resistance to contamination.

It is noteworthy that different nutritional and processing factors, cultivation conditions and growth phases are likely to affect the fatty acid composition of microalgae. In fact, the chain length and degree of unsaturation can change biofuel properties in relation to storage life, combustion characteristics and fluidity (Jesus Ramos et al., 2009).

Thus, a multicriterion-based strategy ought to be considered when selecting a specific wild microalga strain, including a balance of the growing rate and the lipid quantity and quality,

referred to a biomass basis, and focused on the actual distribution of fatty acid within acylglycerols.

Table 1.1. Lipid content and productivity of freshwater and marine species (Mata et al., 2010).

Marine and freshwater microalgae species	Lipid content (% dry weight biomass)	Lipid productivity (mg/L/day)	Volumetric productivity of biomass (g/L/day)
<i>Ankistrodesmus</i> sp.	24.0–31.0	–	–
<i>Botryococcus braunii</i>	25.0–75.0	–	0.02
<i>Chaetoceros muelleri</i>	33.6	21.8	0.07
<i>Chaetoceros calcitrans</i>	14.6–16.4/39.8	17.6	0.04
<i>Chlorella emersonii</i>	25.0–63.0	10.3–50.0	0.036–0.041
<i>Chlorella protothecoides</i>	14.6–57.8	1214	2.00–7.70
<i>Chlorella sorokiniana</i>	19.0–22.0	44.7	0.23–1.47
<i>Chlorella vulgaris</i>	5.0–58.0	11.2–40.0	0.02–0.20
<i>Chlorella</i> sp.	10.0–48.0	42.1	0.02–2.5
<i>Chlorella pyrenoidosa</i>	2.0	–	2.90–3.64
<i>Chlorella</i>	18.0–57.0	18.7	–
<i>Chlorococcum</i> sp.	19.3	53.7	0.28
<i>Cryptothecodium cohnii</i>	20.0–51.1	–	10
<i>Dunaliella salina</i>	6.0–25.0	116.0	0.22–0.34
<i>Dunaliella primolecta</i>	23.1	–	0.09
<i>Dunaliella tertiolecta</i>	16.7–71.0	–	0.12
<i>Dunaliella</i> sp.	17.5–67.0	33.5	–
<i>Ellipsosidion</i> sp.	27.4	47.3	0.17
<i>Euglena gracilis</i>	14.0–20.0	–	7.70
<i>Haematococcus pluvialis</i>	25.0	–	0.05–0.06
<i>Isochrysis galbana</i>	7.0–40.0	–	0.32–1.60
<i>Isochrysis</i> sp.	7.1–33	37.8	0.08–0.17
<i>Monodus subterraneus</i>	16.0	30.4	0.19
<i>Monallanthus salina</i>	20.0–22.0	–	0.08
<i>Nannochloris</i> sp.	20.0–56.0	60.9–76.5	0.17–0.51
<i>Nannochloropsis oculata</i>	22.7–29.7	84.0–142.0	0.37–0.48
<i>Nannochloropsis</i> sp.	12.0–53.0	37.6–90.0	0.17–1.43
<i>Neochloris oleoabundans</i>	29.0–65.0	90.0–134.0	–
<i>Nitzschia</i> sp.	16.0–47.0	–	–
<i>Oocystis pusilla</i>	10.5	–	–
<i>Pavlova</i> sp.	30.9	49.4	0.16
<i>Pavlova lutheri</i>	35.5	40.2	0.14
<i>Phaeodactylum tricornutum</i>	18.0–57.0	44.8	0.003–1.9
<i>Porphyridium cruentum</i>	9.0–18.8/60.7	34.8	0.36–1.50
<i>Scenedesmus obliquus</i>	11.0–55.0	–	0.004–0.74
<i>Scenedesmus quadricauda</i>	1.9–18.4	35.1	0.19
<i>Scenedesmus</i> sp.	19.6–21.1	40.8–53.9	0.03–0.26
<i>Skeletonema</i> sp.	13.3–31.8	27.3	0.09
<i>Skeletonema costatum</i>	13.5–51.3	17.4	0.08
<i>Spirulina platensis</i>	4.0–16.6	–	0.06–4.3
<i>Spirulina maxima</i>	4.0–9.0	–	0.21–0.25
<i>Thalassiosira pseudonana</i>	20.6	17.4	0.08
<i>Tetraselmis suecica</i>	8.5–23.0	27.0–36.4	0.12–0.32
<i>Tetraselmis</i> sp.	12.6–14.7	43.4	0.30

In addition, it must be considered the weakness in responding to environmental disturbances (temperature, nutrient input and light, as well as competition with other microalga and/or bacterial species). Nutrient preference and rate of uptake, in particular CO₂, as well as nitrogen and phosphorus, are especially relevant when carbon sequestration and upgrade of brackish waters and agricultural effluents are sought; biochemical characteristics are also important, from a process perspective, in order to facilitate the biomass downstream processing (biomass harvesting, cell lysis, oil extraction and purification of lipids).

However, after decision on the most appropriate microalga species, photo-biological formulae are still to be optimized for that species, and cost-effective cultivation apparatuses that can precisely deliver the formula to each individual cell are still to be built.

1.2.5. Algal nutrition and metabolism

Microalgae are naturally designed to screen their environment for suitable nutrients and energy, to store them, and to optimize the efficiency of resource utilization. For biomass growth, microalgae depend critically on a sufficient supply of a carbon source and of light to carry out photosynthesis. However, several types of metabolism can be performed, with metabolic shifts being possible in response to changes in environmental conditions, including pH – which will affect growth (Mata et al., 2010).

Phototrophic cultivation occurs when the microalgae use light, for instance sunlight, as the energy source, and inorganic carbon dioxide as the carbon source to form chemical energy through photosynthesis (Huang et al., 2010). This is the most commonly used cultivation condition for microalgae growth (Gouveia and Oliveira, 2009; Mandal and Mallick, 2009; Yoo et al., 2010). The major advantage of using autotrophic cultivation to produce microalgal oil is the consumption of CO₂ as carbon source for cell growth and oil production. However, when CO₂ is the only carbon source, the microalgae cultivation site should be close to factories or power plants which can supply a large amount of CO₂ for microalgal growth. Moreover, compared to other types of cultivation, the contamination problem is less severe when using autotrophic growth. Therefore, outdoor scale-up microalgae cultivation systems (such as open ponds and raceway ponds) are usually operated under phototrophic cultivation conditions (Mata et al., 2010).

Some microalgae species not only can grow under phototrophic conditions, but are also able to use organic carbon under dark conditions, just like bacteria. The situation when microalgae use organic carbon as both the energy and carbon source is called heterotrophic cultivation (Chojnacka and Noworyta, 2004). This type of cultivation could avoid the problems associated with limited light that hinder high cell density in large scale photobioreactors during phototrophic cultivation (Huang et al., 2010). Higher biomass production and productivity could be obtained from using heterotrophic cultivation. Some microalgae species show higher lipid content during heterotrophic growth, and a 40% increase in lipid content was obtained in *Chlorella protothecoides* by changing the cultivation condition from phototrophic to heterotrophic (Xu et al., 2006). Microalgae can assimilate a variety of organic carbon sources for growth, such as glucose, acetate, glycerol, fructose, sucrose, lactose, galactose, and mannose (Liang et al., 2009). The highest lipid productivity (3700 mg/L d) was reported by (Xiong et al., 2008) using a 5-L fermenter operated with an improved fed-batch culture strategy with glucose. Using heterotrophic growth gives much higher lipid productivity, as the highest lipid productivity from heterotrophic cultivation is nearly 20 times higher than that obtained under phototrophic cultivation.

Mixotrophic cultivation is when microalgae simultaneously use light energy for photosynthesis and both organic compounds and inorganic carbon (CO₂) as a carbon source for growth. It

means that microalgae are able to live under either phototrophic or heterotrophic conditions, or both (Mata et al., 2010). Compared with phototrophic and heterotrophic cultivation, mixotrophic cultivation is rarely used in microalgal oil production, although, for certain species, the presence of light stimulates an higher uptake of substrate (Perez-Garcia et al., 2011).

Although oil production of microalgae is strain-dependent, it seems that mixo- and heterotrophic growth could give much higher oil productivity than other cultivation conditions, an approach that has attracted considerable interest (Liang et al., 2009).

However, heterotrophic culture can get contaminated very easily, especially in open systems, causing problems in large-scale production. In particular, the sugar based heterotrophic system frequently suffers from problems with contamination. In addition, the cost of an organic carbon source is also a major concern not only from the commercial aspect, but also considering that the exploitation of organic substrate like glucose has the same limits of 1st generation biofuels.

Phototrophic cultivation is most frequently used, it is easiest to scale up and is promising because microalgae could consume CO₂ from flue gases of factories and convert it to oil. However, the oil productivity of this approach is usually markedly lower than that of heterotrophic cultivation, due mainly to slower cell growth and lower biomass productivity (Chen et al., 2011), a behavior that is indeed species-dependent. On the other hand, the lower cost for scaling-up of phototrophic cultivation means that this method is very attractive anyway.

1.3. Culture systems

The interest for phototrophic organisms lies in their potential utilization, similarly to heterotrophic microorganisms, to produce biomass for food, feed and fine chemicals, using solar energy. The origins of applied phycology most probably date back to the establishment of a culture of *Chlorella* by Beijerinck, in 1890 (Tomaselli, 2003). Even today *Chlorella* takes up the first place in the commercial use of these microorganisms. Utilizing microalgal lipids has been of interest since Harder and von Witsch (1942) proposed the mass cultivation of diatoms to obtain urgently needed fat in World War II.

However, producing microalgal biomass is generally more expensive than growing crops. Photosynthetic growth requires light, carbon dioxide, water and inorganic salts. Temperature must remain generally within 20 to 30 °C. To minimize expense, biodiesel production must rely on freely available sunlight, despite daily and seasonal variations in light levels. Growth medium must provide the inorganic elements that constitute the algal cell. Essential elements include nitrogen (N), phosphorus (P), iron and in some cases silicon (Chisti, 2007). The

common methods of large-scale production of microalgae are raceway ponds and photobioreactors (Molina Grima et al., 1999).

1.3.1. Open ponds

A raceway pond is made of a closed loop recirculation channel that is typically about 0.3 m deep. Mixing and circulation are produced by a paddlewheel: flow is guided around bends by baffles placed in the flow channel and it operates all the time to prevent sedimentation.

Raceway ponds for mass culture of microalgae have been used since the 1950s (Spolaore et al., 2006). Production of microalgal biomass for making biodiesel has been extensively evaluated in raceway ponds in studies sponsored by the United States Department of Energy (Sheehan et al., 1998). Raceways are perceived to be less expensive than photobioreactors, because they cost less to build and operate. Although raceways are low-cost, they have a low biomass productivity compared with photobioreactors. In raceways, cooling is achieved by evaporation only and temperature fluctuates within a diurnal cycle and seasonally, leading to a significant evaporative water loss. In addition, raceways use carbon dioxide much less efficiently than photobioreactors and productivity is affected by contamination with unwanted algae and microorganisms that feed on algae. The biomass concentration remains low because raceways are poorly mixed and cannot sustain an optically dark zone. However, extensive experience exists on operation and engineering of raceways. The most recent raceway-based biomass production facility will be built by Seambiotic's Algae Plant (Israel), that will use flue gas in China (Ben-Amotz et al., 2011). Though originally scheduled to be operational in 2010, the plant should be opened in September 2011, although not recent informations are still available. During the "1st International Conference on Algal Biomass, Biofuels, and Bioproducts" (St. Louis, July 2011), Ben-Amotz explained that the choice to build the plant in China was due to several reasons. First, finding additional land on the coast of Israel would have been prohibitively expensive. Additionally, the alterations needed at a power plant to pump the flue gas into the algae ponds were such that most operators would not want to have to deal with the complexity. Lastly, the cost of the materials, land, and labour in China made perfect sense to build the plant there. The finished plant will be approximately 10 hectares in size, making it the largest plant of its kind in the world. The author believes that this plant will be able to produce algae biomass to convert into fuel at prices competitive with traditional fuel by 2012.



Fig 1.5: Open ponds (from <http://climatetechwiki.org/technology/algae>)

1.3.2. Closed systems

Unlike open raceways, photobioreactors (PBR) permit essentially single-species culture of microalgae for prolonged durations. PBRs, defined as reactors in which phototrophs (microbial, algal or plant cells) are grown or used to carry out a photobiological reaction, have been successfully applied for producing large quantities of microalgal biomass (Molina Grima et al., 1999; Carvalho et al., 2006). However, there are no PBR examples at industrial scale.

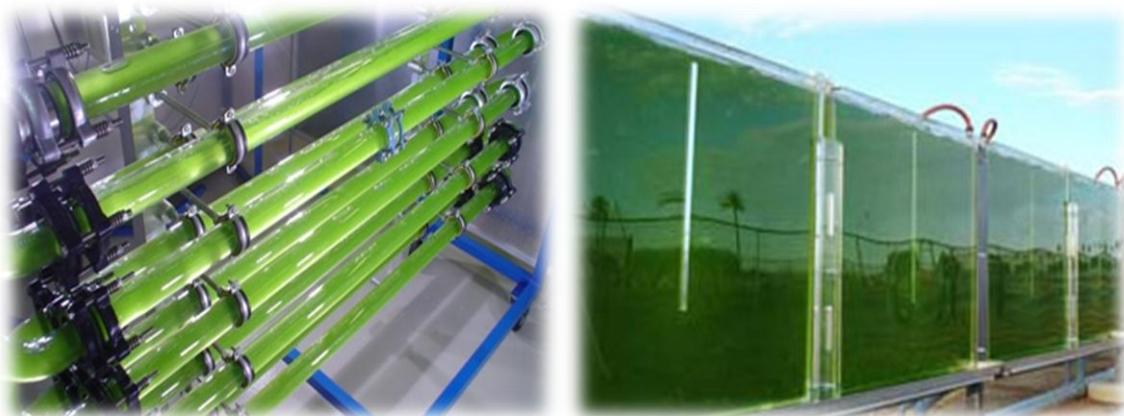


Fig 1.6. Closed photobioreactors, as tubular reactor or panel reactor.

PBRs are flexible systems that can be optimized according to the biological and physiological characteristics of the algal species being cultivated, allowing to cultivate algal species that cannot be grown in open ponds. On a PBR, direct exchange of gases and contaminants (e.g. microorganisms, dust) between the cultivated cells and the atmosphere are limited or not allowed by the reactor's walls. Also, a great proportion of light does not impinge directly on the culture surface but has to cross the transparent reactor walls. Depending on their shape or design, PBRs have several advantages over open ponds: offer better control over culture conditions and growth parameters (pH, temperature, mixing, CO₂ and O₂), prevent evaporation, reduce CO₂ losses, they allow to attain higher microalgae densities or cell

concentrations, i.e. higher volumetric productivities, offer a safer and more protected environment, preventing contamination or minimizing invasion by competing microorganisms (Mata et al., 2010).

PBRs can be designed as tubular or flat panel reactors that are usually made of plastic or glass. In tubular PBRs the tube diameter is limited because light does not penetrate too deeply in the dense culture broth that is necessary for ensuring a high biomass productivity of the photobioreactor.

Despite their advantages, it is not clear if PBRs could have a significant impact in the near future on any product or process that can be attained in large outdoor raceway ponds. PBRs suffer from several drawbacks that need to be considered and solved. Their main limitations include: overheating, bio-fouling, oxygen accumulation, difficulty in scaling up, high building and operating costs and cell damage by shear stress and deterioration of material used for the photo-stage. The cost of biomass production in PBRs may be one order of magnitude higher than in open ponds (Mata et al., 2010). Anyway, the higher cell concentration and the higher productivity achieved in PBRs might compensate for their higher capital and operating costs, although some issues have no solution yet.

1.4. Environmental applications – integrated process

The potential of microalgae as a source of renewable energy has received considerable interest, but microalgal biofuel production must to be economically viable in order to become sustainable. Consequently, further optimization of mass culture conditions are needed. Wastewaters derived from municipal, agricultural and industrial activities potentially provide cost-effective and sustainable means of algal growth for biofuels with the potential of combining wastewater treatment by algae, such as nutrient removal, with biofuel production (Pittman et al., 2011).

Thus, the combination of the three roles of microalgae (CO₂ fixation, wastewater treatment and biofuel production) has the potential to maximize the impact of microalgal biofuel (Gong and Jiang, 2011). However, a number of crucial research gaps remain that must be overcome to achieve full-scale operation, including improved algal growth and nutrient uptake rates, and integration of biosystems with waste gas, wastewater and water reclamation systems (Kumar et al., 2010).

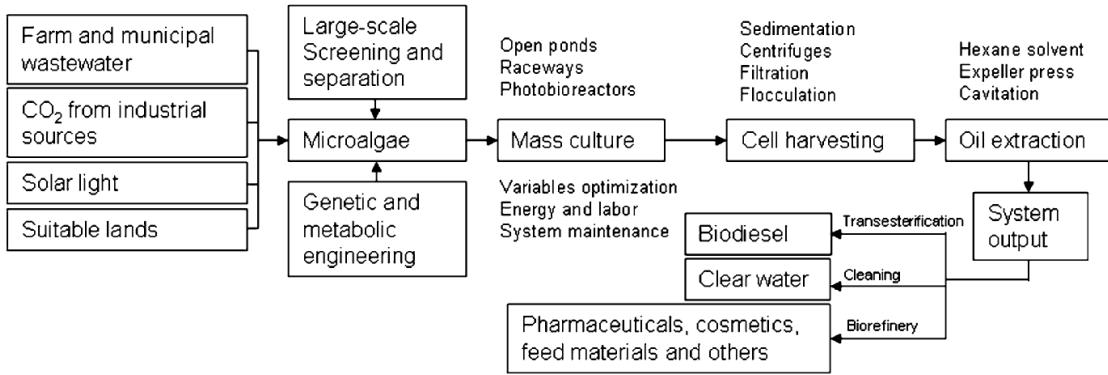


Figure 1.7. An integrated system for large-scale biodiesel production with microalgae as feedstock. This system aims at reducing the overall cost for biodiesel production, and emphasizes on the integrative utilization of by-products and residual compounds both from economic and environmental viewpoints (Gong and Jiang, 2011).

1.4.1. Flue gas CO₂ emissions as microalgae nutrient

An increase in atmospheric CO₂, derived from fossil fuel combustion, poses great challenges to world development sustainability. The Kyoto protocol was based on the need of reducing greenhouse gas emissions, especially aimed at lowering the amount of carbon dioxide released. Technologies on the mitigation of carbon dioxide emission have been developed including physicochemical absorbents, injection into deep oceans and geological formations, and enhanced biological fixation (Kumar et al., 2010, Ho et al., 2011). The path of carbon in the photosynthesis has been considered as a tool to fix carbon dioxide from flue gas (with 10–20% CO₂) (Hsueh et al., 2009). One of the key advantages of using microalgae lies in the ability of some microalgal species to tolerate high CO₂ content in feeding air streams, allowing efficient capture of CO₂ from high-CO₂ streams such as flue gases and flaring gases (Li et al., 2008). In comparison to terrestrial plants, which typically absorb CO₂ from the atmosphere (containing only 0.03–0.06% CO₂), the benefit of microalgae is evident in terms of CO₂ mitigation. For instance, it was reported that using an outdoor cultivation of *Chlorella* sp. in a 55 m² culture area photobioreactor, with flue gas containing 6–8% by volume of CO₂, it was possible to achieve 10–50% CO₂ mitigation, and the residual NO₂ and NO in the flue gas was found not to affect algal growth (Li et al., 2008).

1.4.2. Nitrogen and phosphorus supply from wastewaters

The treatment of wastewater is nowadays an open issue in many parts of the world. The major disadvantages associated with current wastewater treatment practices are related to operational cost, high energy requirements and maintenance requirements including production of large volumes of sludge.

On the other hand, phytoremediation applied to the removal of nutrients from animal wastewater and other high organic content wastewater is a field with a great potential and demand, considering that surface and underground water bodies in several regions of the

world are suffering of eutrophication (Olguin, 2010). However, the development of more efficient nutrient removal algal systems requires further research in key areas. Algae growth rate controls directly and indirectly the nitrogen and phosphorus removal efficiency. Thus, maximum algae productivity is required for effective nutrient removal and must be considered as a key area of research.

In addition to the apparent benefit of combining microalgal biomass, and therefore biofuel, production with wastewater treatment, successful implementation of this strategy would allow to minimize the use of freshwater, another precious resource especially for dry or populous countries, for biofuel production. Extensive works have been conducted to explore the feasibility of using microalgae for wastewater treatment, especially for the removal of nitrogen and phosphorus from effluents (Pittman et al, 2011; Chinnasamy et al., 2010; Kong et al., 2010).

A major concern associated with using wastewater for microalgae cultivation is contamination. This can be managed by adding appropriate pretreatment technologies to remove sediment and to sterilize the wastewater, but this could annul the economical feasibility of the process.

1.5. Academic research and industrial practice: the current scenario

In the recent years, microalgae are receiving a growing attention for their possible exploitation in biofuel production. The academic research is widely active and new research groups start to work in this fields every year. In Europe, several universities are involved in microalgae research projects: universities in the United Kingdom which are working on producing oil from algae include Cambridge University and Imperial College London. In Spain, it is also relevant the research carried out by the CSIC's Instituto de Bioquímica Vegetal y Fotosíntesis (Microalgae Biotechnology Group, Seville) as well as University of Almeria, that is focusing on photobioreactor design. In the Netherlands, Wageningen University and Research Centre has strong expertise in research on microalgae in the fields of genomics, metabolomics, physiology, reactor engineering and biorefinery. They are also widely working on applications of microalgae, varying from the use of residues, production of fine chemicals, food ingredients and biofuels.

Concerning the situation in the USA, the interest in microalgae biology and cultivation is growing, in particular due to the support of the United States Department of Energy (DOE). DOE is a Cabinet-level department of the US government concerned with the United States policies regarding domestic energy production and it sponsors basic and applied scientific research. The Aquatic Species Program, launched in 1978, was a research program funded by DOE which was tasked with investigating the use of algae for the production of energy. The program initially focused on the production of hydrogen, shifting primary research to studying oil production in 1982. From 1982 until its end in 1996, the majority of the program research

was focused on the production of transportation fuels, notably biodiesel, from algae. In 1995, as part of overall efforts to lower budget demands, DOE decided to end the program. Research stopped in 1996 and staff began compiling their research for publication. In recent years, DOE is supporting the major institutions and facilities which are working on microalgae, by starting the National Alliance for Advanced Biofuels and Bioproducts (NAABB). The overall goal of the NAABB consortium is to produce new technologies that can be implemented by commercial partners, and others, developing the algal biofuel industry. In order to achieve this goal, the program incorporates major objectives in Algal Biology, Cultivation, Harvesting/Extraction and Conversion to fuels, Coproduct development, Economic/energy-balance modeling and resource management components that will establish the technologies viability for overall sustainability. Several US universities are involved in this project and are working on producing oil from algae. In addition, some oceanographic Institutions are focusing on the wastewater utilization from domestic and industrial sources containing rich organic compounds that are being used to accelerate the growth of algae.

As interest in algal biofuels re-emerges globally, many industry players are actively pursuing R&D ventures, operating pilot scale facilities and seeking investment support. Over time, the market will likely experience some consolidation as various current technologies exit the market due to the lack of cost-competitiveness and production efficiency. In fact, while it is generally acknowledged that it is possible to produce fuels from algae cultures, process development is still at early stage. Several pilot-scale plants have been successfully tested, but in large scale, there is to date no facility generating microalgal biofuels effectively in terms of both energy and financial cost. The largest operating plants use open pond systems to grow algae for food, feed and cosmetic purposes. The productivity of these facilities is comparatively low, but the simple design and the high value of the products (up to several thousand dollars per kilogram) make these processes profitable (Morweiser et al., 2010). In addition, there is a lack of fundamental information needed to rationally optimize the performance of existing bioreactors. Novel bioreactor configurations and designs are also needed that promote microalgal growth, characterized by volumetric productivities at least one order of magnitude above those of conventional open pond facilities (Kumar et al., 2010). Thus, algal fuels are not commercial yet, but their economic outlook is promising. Dozens of startup companies are attempting to commercialize algal fuels (Table 1.2) (Chisti and Yan, 2011). The most important one are Sapphire Energy (San Diego), that has produced green crude from algae, and Solazyme (South San Francisco, California) which has produced a fuel suitable for powering jet aircrafts by algae.

Table 1.2. Examples of startup companies attempting to commercialize algal fuels (Chisti and Yan, 2011).

Company	Location	Web site
Algenol Biofuels	Bonita Springs, FL, USA	www.algenolbiofuels.com
Aquaflow	Nelson, New Zealand	www.aquaflowgroup.com
Aurora Algae, Inc.	Hayward, CA, USA	www.aurorainc.com
Bioalgene	Seattle, WA, USA	www.bioalgene.com
Bionavitas, Inc.	Redmond, WA, USA	www.bionavitas.com
Bodega Algae, LLC	Boston, MA, USA	www.bodegaalgae.com
LiveFuels, Inc.	San Carlos, CA, USA	www.livefuels.com
PetroAlgae Inc.	Melbourne, FL, USA	www.petroalgae.com
Phyco Biosciences	Chandler, AZ, USA	www.phyco.net
Sapphire Energy, Inc.	San Diego, CA, USA	www.sapphireenergy.com
Seambiotic Ltd.	Tel Aviv, Israel	www.seambiotic.com
Solazyme, Inc.	South San Francisco, CA, USA	www.solazyme.com
Solix Biofuels, Inc.	Fort Collins, CO, USA	www.solixbiofuels.com
Synthetic Genomics Inc.	La Jolla, CA, USA	www.syntheticgenomics.com

In addition, there are international organization with the aim of supervising the research on microalgae and integrate academic and private knowledge. The Algal Biomass Organization (ABO) is formed by Boeing Commercial Airplanes, A2BE Carbon Capture Corporation, National Renewable Energy Labs, Scripps Institution of Oceanography, Benemann Associates, Mont Vista Capital and Montana State University and organizes the most important scientific congress on this topic every year.

In Europe, the European Algae Biomass Association (EABA) is the association representing both research and industry in the field of algae technologies, currently with 79 members. The association is headquartered in Florence, Italy. The general objective of EABA is to promote mutual interchange and cooperation in the field of biomass production and use, including biofuels uses and all other utilizations. It is aimed to creating, developing and maintaining solidarity and links between its members and at defending their interests at European and international levels. Its main target is to act as a catalyst for fostering synergies among scientists, industrialists and decision makers in order to promote the development of research, technology and industrial capacities in the field of algae.

The industrial situation in Italy is still at an early stage. In 2007, researchers at EniTecnologie in Italy conducted a field experiment of CO₂ uptake by algae in a raceway pond: *Tetraselmis suecica*, a marine water algae was used for that study (www.powerplantccs.com). The strains were supplied with CO₂ from natural gas turbine flue gas. EniTecnologie reported growth rates as mass of dry algae produced each day per square meter of raceway, the productivity ranged between 10 and 30 g m⁻² day⁻¹. The CO₂ uptake represents roughly half the weight of the dry algae, or 5 to 15 g CO₂ m⁻² day⁻¹. The biomass would be harvested and then fermented by anaerobic digestion to methane to replace a fraction of the natural gas, with the residual

sludge, containing most of the N, P and other nutrients, recycled back to the cultivation ponds. A 700 ha system was designed to be able to mitigate 15% of the annual CO₂ emissions from a 500 MW NGCC power plant. This plant is apparent operating at the Gela Refinery premises, but its characteristics and performances are not to be disclosed.

1.6. Biofuels from microalgae: final remarks

Fuels from algae certainly look promising. They may already be viewed as competitive with respect to petroleum fuels, if the full environmental impact of these is taken into consideration. Issues of climate change may force us to move beyond petroleum long before it runs out (Chisti and Yan, 2011). The industrial viability of microalga-based biofuels hinges upon the economics underlying the process; whatsoever advances might arise in terms of technological innovations, the market will not exhibit an enthusiasm for funding capital-intensive energy projects unless the risk-return ratio is acceptable (Singh and Gu, 2010).

The global cost associated with biodiesel production by microalgae may be split into the partial costs due to biomass growth, harvesting (including dewatering and concentration of biomass to a suitable level for further processing), oil extraction and oil transesterification. Furthermore, the traditional project costs of engineering, licensing, infrastructure build up, equipment purchase, installation and integration, and contractor fees are also to be considered. In terms of other operating and maintenance costs, expenses for nutrients (generally nitrogen and phosphorus sources), enriched CO₂ supply, water replenishment due to evaporative losses, other power utilities, components replacement and for labour are to be considered as well to allow an accurate evaluation (Singh and Gu, 2010).

Since microalga production systems are a rather complex combination of several subsets (i.e. production, harvesting, extraction and drying systems), reducing the associated number of steps is thus crucial to lower costs.

Although microalgae offer a number of advantages, a sustainable algal biofuel industry is at least one or two decades away from maturity, and no commercial scale operations currently exist. Several barrier must first be overcome before algal biofuel s can compete with traditional petroleum-based fuels. Production chains with net energy output need to be identified and continuous R&D is needed to reduce the cost in all segments of the production spectrum. In the longer term, genetic engineering will likely have the greatest impact on the feasibility of algal biofuels. Advances in methods of separating the algal biomass from the water and extraction of the oil from the biomass, will improve the prospects of algal oil. For example, through genetic engineering, the cells of certain photosynthetic microorganisms have been coaxed into secreting the oil that would normally be retained within the cell, thus simplifying oil recovery. Algal species engineered to use atmospheric nitrogen instead of

nitrogen fertilizers that are now required, will be a great step forward, as production of nitrogen fertilizers is heavily dependent of petroleum (Chisti and Yan, 2011).

To effectively address current bottlenecks associated with microalgal biodiesel, a leap in both fundamental knowledge and technological applicability is required. After this has been achieved, microalga-mediated manufacture of biodiesel will become a fully competitive process (Malcata, 2011).

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CHAPTER 2

EXPERIMENTAL SET UP AND SCREENING OF SPECIES

In this chapter the set up of materials, methods and experimental apparatus are reported. Some of the most common cell culture methods were used in order to measure growth kinetics of microalgae, and two techniques were optimized for lipid content determination. In addition, experimental apparatus was set up for growth conditions optimization, non-limiting CO₂ supply and effect of light on growth experiments. Several microalgal species were screened, in order to select the most promising ones from an industrial point of view.

2.1. Materials and methods

This section of the thesis gives a detailed account of the procedure we followed to perform the experiments presented and discussed in this and further chapters. In particular, Nile Red staining and Soxhlet extraction were optimized in this work for lipid determination.

2.1.1. *Microalgae and media composition*

Several microalgae species were used in this work (table 2.1): *Botryococcus braunii* 807.1, *Chlamydomonas reinhardtii* WT8B+, *Chlorella vulgaris* emerson3, *Scenedesmus quadricauda*, *Nannochloropsis limnetica* and *Nannochloropsis salina* 40.85. All of them were screened for their growth characteristics.

C. reinhardtii, *B. braunii*, *C. vulgaris*, *N. limnetica* and *S. quadricauda* are freshwater species, and were grown in BG11 medium (see table 2.2 for media composition (Rippka et al., 1979). *N. salina*, a marine species, was cultured in sterilized sea salts 22g/L solution enriched with f/2 Guillard solution (table 2.2) as described by (Guillard and Ryther, 1962).

Table 2.1. Microalgal strains

Species	Strain	Source
<i>Botryococcus braunii</i>	807.1	SAG - Culture Collection of Algae at Goettingen
<i>Chlamydomonas reinhardtii</i>	WT8B+	University of Padova
<i>Chlorella vulgaris</i>	emerson3	CNR Firenze
<i>Scenedesmus quadricauda</i>	-	CNR Firenze
<i>Nannochloropsis limnetica</i>		SAG - Culture Collection of Algae at Goettingen
<i>Nannochloropsis salina</i>	40.85	SAG - Culture Collection of Algae at Goettingen

2.1.2. Inoculum maintenance

Monospecific cultures were inoculated in small volumes under controlled conditions, in liquid or solid media (Petri dishes with agar gels). These pre-inoculums were maintained in axenic conditions and were utilized as batch growth curves inoculums.

Table 2.2. BG11 and f/2 media composition

BG11		f/2 Guillard	
Component	Concentration (M)	Component	Concentration (M)
NaNO ₃	1,7 x 10 ⁻²	NaNO ₃	8,82 x 10 ⁻⁴
K ₂ HPO ₄	2,2 x 10 ⁻⁴	NaH ₂ PO ₄ H ₂ O	3,62 x 10 ⁻⁵
MgSO ₄ ·7H ₂ O	3,0 x 10 ⁻⁵	Na ₂ SiO ₃ 9H ₂ O	1,06 x 10 ⁻⁴
CaCl ₂ ·2H ₂ O	2,0 x 10 ⁻⁴	FeCl ₃ 6H ₂ O	1,17 x 10 ⁻⁵
Citric Acid·H ₂ O	3,0 x 10 ⁻⁵	Na ₂ EDTA 2H ₂ O	1,17 x 10 ⁻⁵
Ammonium Ferric Citrate	2,0 x 10 ⁻⁵	CuSO ₄ 5H ₂ O	3,93 x 10 ⁻⁸
Na ₂ EDTA·2H ₂ O	2,0 x 10 ⁻⁶	Na ₂ MoO ₄ 2H ₂ O	2,60 x 10 ⁻⁸
Na ₂ CO ₃	1,8 x 10 ⁻⁴	ZnSO ₄ 7H ₂ O	7,65 x 10 ⁻⁸
H ₃ BO ₃	4,6 x 10 ⁻⁵	CoCl ₂ 6H ₂ O	4,20 x 10 ⁻⁸
MnCl ₂ ·4H ₂ O	9,0 x 10 ⁻⁶	MnCl ₂ 4H ₂ O	9,10 x 10 ⁻⁷
ZnSO ₄ ·7H ₂ O	7,7 x 10 ⁻⁷	Thiamine HCl (vit. B ₁)	2,96 x 10 ⁻⁷
Na ₂ MoO ₄ ·2H ₂ O	1,6 x 10 ⁻⁶	Biotin (vit. H)	2,05 x 10 ⁻⁹
CuSO ₄ ·5H ₂ O	3,0 x 10 ⁻⁷	Cyanocobalamin (vit. B ₁₂)	3,69 x 10 ⁻¹⁰
COCl ₂ ·6H ₂ O	2,5 x 10 ⁻⁴		
HEPES	1,0 x 10 ⁻²		

2.1.3. Growth analysis

Growth is defined as an increase in living substance, usually the number of cells for unicellular microorganisms or the total mass of cells for multicellular organisms. In unicellular microalgae the cell size generally doubles and then the cell divides into two daughter cells which will then increase in size. The cell cycle in eukaryotic algae involves two phases: mitosis and interphase. During the interphase the cell grows and all cellular constituents increase in number so that

each daughter cell will receive a complete set of the replicated DNA molecule and sufficient copies of all other constituents and organelles. During the mitosis the nuclear division occurs. Microbial growth is influenced by several chemical and physical conditions. As the substrate concentration or other factors become limiting, or toxic metabolites accumulate, the growth rate decreases. In this growth phase, the production of secondary metabolites often takes place. As long as there is consumption of stored material the organism remains viable. When energy is no longer produced for cell maintenance, the cell declines and finally dies. All the species considered showed a typical sigmoidal growth curve. Specific growth rate determination was calculated during the exponential growth phase, when substrates are not limiting. The growth rate is a measure of the change in cell number or cell mass per unit time. Growth experiments were performed in Erlenmeyer flasks (batch system). Each autotrophic batch cultivation (250mL) was carried out in duplicate. The medium and flasks were sterilized in an autoclave for 20 min at 121 °C in order to prevent any contamination. The growth temperature was 24±1°C, with artificial lighting (fluorescent tubes) under a continuous photon flux density of $120\pm10\mu\text{E m}^{-2}\text{ s}^{-1}$, measured by a photoradiometer (LI-COR, Model LI-189). The duration of the experiment depended on the growth rate. Algal growth kinetics was measured by daily changes in optical density (measured at 750 nm, by a Perkin Elmer-Lambda Bio 40 spectrophotometer) and cells number (by using a Burker cells counter). Only during the logarithmic growth phase, cells number was related to optical density: when the culture reaches the stationary phase, OD increases while the number of cells remains constant.

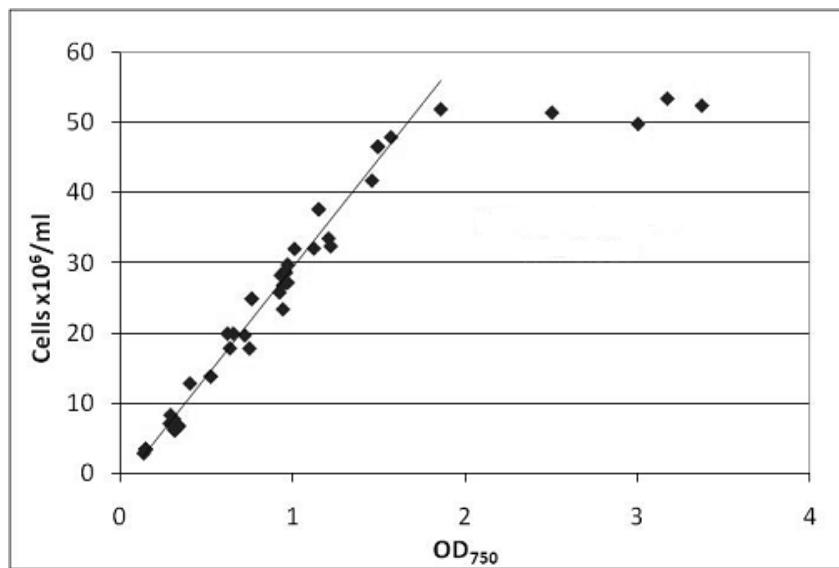


Figure 2.1 Linear correlation between OD and number of cells during exponential phase of growth for *N. salina*. The correlation equation is $y=3x10^6-2x10^6$, $R^2=0.98$.

For dry weight (DW) determinations, cells were harvested with a 0.22 µm filter. DW was measured gravimetrically upon drying the filters at 100°C for 4 h in a laboratory oven. DW data are reported as g/L.

The elemental chemical analysis of microalgal biomass was performed by Chelab Srl. The elemental analysis, CHN analysis, is accomplished by a combustion analysis. In this technique, a sample is burned in an excess of oxygen, and various traps collect the combustion products—carbon dioxide, water, and nitric oxide. The masses of these combustion products can be used to calculate the composition of the unknown sample.

2.1.4. Specific growth rate

The specific growth rate was calculated by the slope of logarithmic phase in terms of number of cells, in the absence of any substrate limitations. Under these conditions the Malthus equation holds:

$$\frac{dX}{dt} = \mu \cdot X \quad (2.1)$$

where X is the cells number, t the time and μ the specific growth rate (t^{-1}) of the culture. The μ defines the fraction of increase in biomass over a unit time, i.e. an increase of biomass per unit time from every unit of existing biomass. Specific growth rate represents the average growth rate of all cells present in a culture, but not necessarily the maximum specific growth rate of the individual cells, as most microbial cultures divide asynchronously. The expression of the rate of microbial growth as a specific growth rate avoids the effect of cell concentration. In figure 2.2 a semi-logarithmic growth curve for *N. salina* is reported.

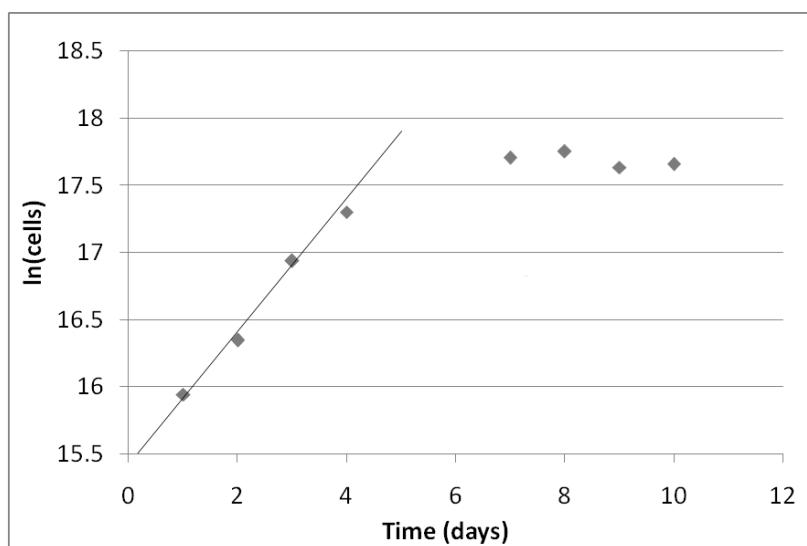


Figure 2.2: semi-logarithmic growth curve of *N. salina* and linear regression of data of exponential growth phase. The correlation equation is $y=0.497x + 15.41$, therefore $\mu=0.497$.

The time required to double the number of viable cells is termed doubling time (t_d). It is also called generation time, as it is the time taken to grow and produce a new generation of cells. The number of cells in an exponential growing culture is:

$$X = X_0 \cdot 2^n \quad (2.2)$$

where X_0 is the number of cells at starting concentration and n is the number of cell divisions, that at time t corresponds to t/t_d .

$$X = X_0 \cdot 2^{t/t_d} \quad (2.3)$$

$$\ln\left(\frac{X}{X_0}\right) = (\ln 2) \cdot t/t_d \quad (2.4)$$

$$\frac{1}{X} \cdot \frac{dX}{dt} = 0,693/t_d \quad (2.5)$$

The relation between doubling time and the specific growth rate is :

$$\mu = 0,693/t_d \quad (2.6)$$

Number of doublings (n) at a time interval t , is determined by the relation t/t_d .

During the exponential growth phase, the growth rate of the cells is proportional to the biomass of cells. When biomass can be measured more accurately than the number of cells, the basic microbial growth equations are often expressed in terms of mass.

2.1.5. Measurement of lipid content by Nile Red staining

The lipid content was determined by staining the algal cell suspension with Nile Red (NR) dye (Chen et al., 2009). The fluorescence was measured using a spectrofluorimeter (OLIS DM45), with excitation wavelength at 488 nm and emission wavelength in the range of 500 and 700 nm. The relative fluorescence of Nile Red for the lipids was obtained after subtraction of the autofluorescence of algal cells and Nile Red alone.

In order to set up the technique, some preliminary experiments were done by changing both dye and cells concentrations in the sample. Different Nile Red concentrations were used: 0.625 µg/ml, 1.25 µg/ml, 1.875 µg/ml, 2.5 µg/ml, at the same cell concentration. Samples were incubated for 10 minutes at 37°C (Chen et al., 2009) and spectra are reported in figure 2.3. As showed in figure 2.3, the fluorescence intensity (IF) increases in the range of dye concentrations between 0.625 µg/ml and 1.85 µg/ml, while the concentration of 2.5 µg/mL seems to be in excess. That concentration is more than enough to detect all lipids present in the sample. For this reason, we selected the concentration of 2.5 µg/mL for further experiments.

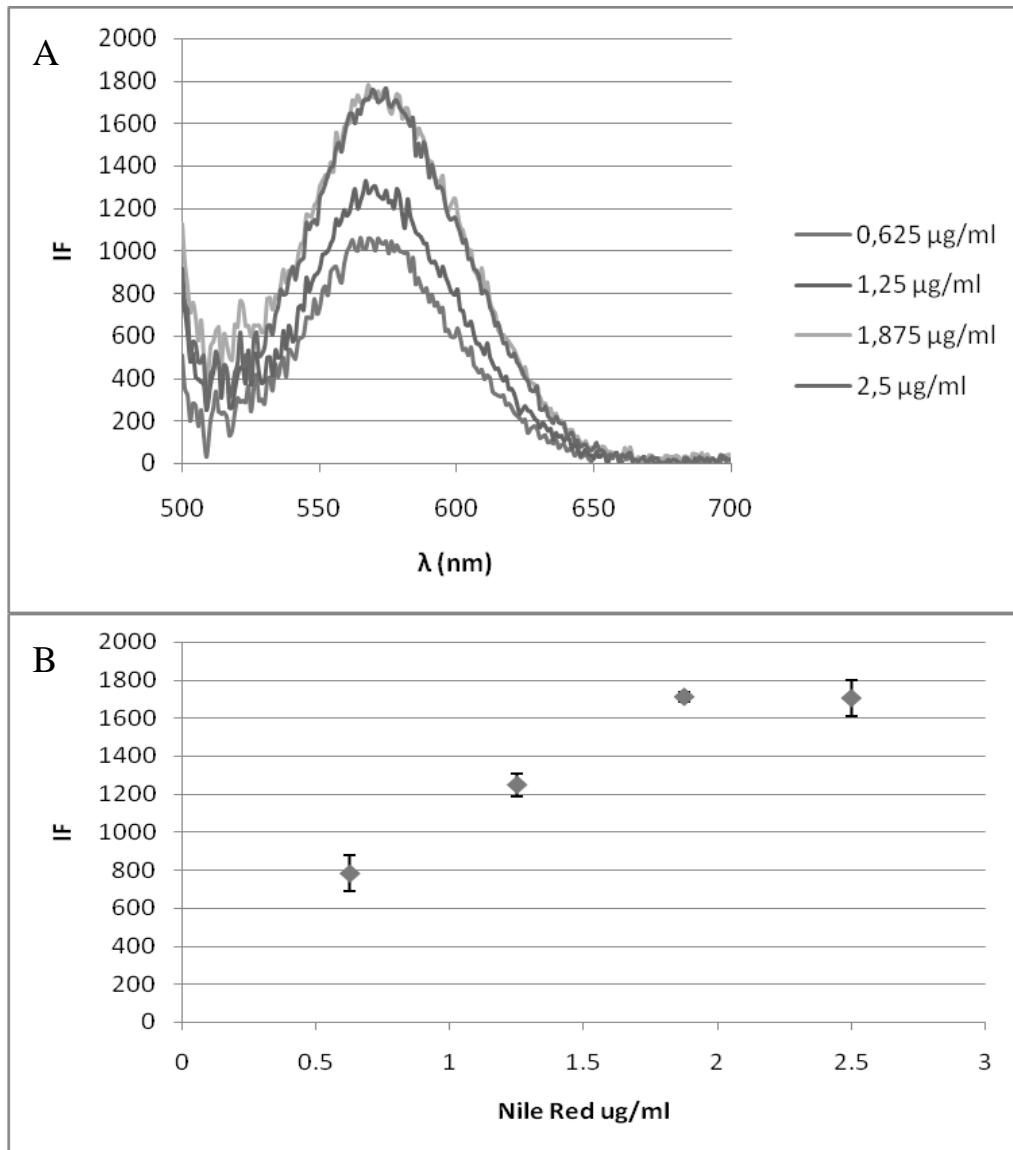


Figure 2.3: fluorescence spectra of *N. salina* stained cells at different dye concentration are reported (figure 2.3A). Average data of maximum IF peak (ranging from 575 to 585 nm) for each concentration (figure 2.3B).

Furthermore, we measured fluorescence spectra at different cell concentrations (ranging from 1 to 6 millions of cells per milliliter), at the same Nile Red concentration of 2,5 $\mu\text{g}/\text{ml}$. As reported in figure 2.4, the IF value is linearly related to the cell concentration, showing that the dye concentration is in excess and can detect all lipids present in the sample. Anyway, we decided to use the precautionary concentration of 2×10^6 cells/ml for further experiments, in case of any higher lipid accumulation.

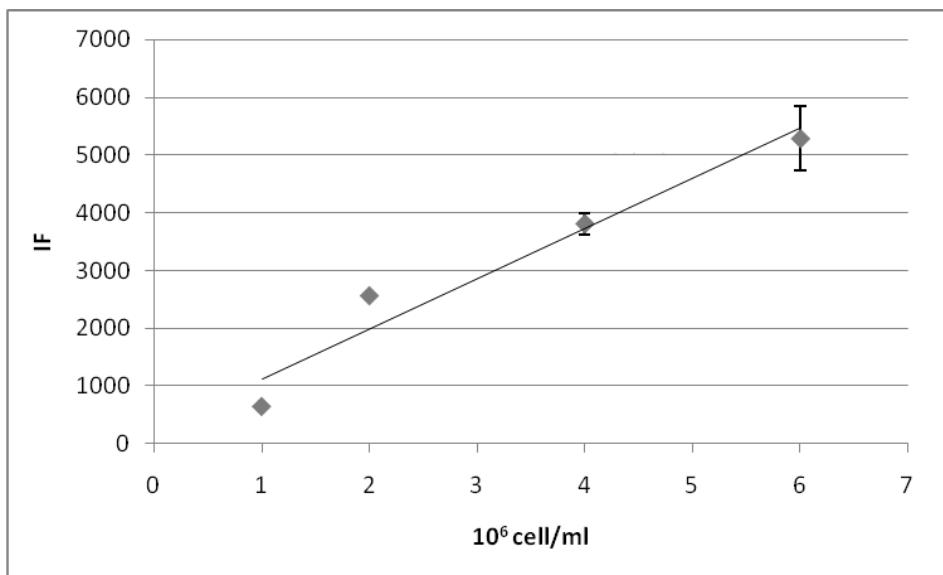


Figure 2.4: Maximum IF of *N.salina* stained samples at different cell concentrations.

2.1.6. Soxhlet extraction of total lipids

IF data were correlated to total lipid content measured by Soxhlet extraction. An algal culture was centrifuged at 5000 rpm for 10 minutes and washed with distilled water, to remove salts from culture media. The biomass was dried in a oven at 60°C. Total lipids were extracted from dried cells using ethanol-hexane (2.5:1 vol/vol) as solvent mixture (Molina Grima et al., 1994) in a Soxhlet apparatus (Figure 2.5).

The biomass is placed inside a thimble made from a filter paper (0.22 µm of porosity), which is loaded into the main chamber of the Soxhlet extractor. The Soxhlet extractor is placed onto a flask containing the extraction solvent. The solvent is heated, so that the solvent vapour travels up a distillation arm, and floods into the chamber housing the thimble of dried biomass. A condenser ensures that solvent vapour turns into liquid and drip back down into the chamber housing the solid material.

The chamber containing the biomass is slowly filled with warm solvent. Total lipids will then dissolve in the warm solvent. When the Soxhlet chamber is almost full, the chamber is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This solvent recirculation was repeated for about 24 hours.

During each cycle, a portion of the non-volatile compound dissolves in the solvent. After many cycles the desired compound is concentrated in the distillation flask. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled.

After extraction the solvent is removed, by a rotary evaporator, yielding the extracted compounds. The lipid mass was measured gravimetrically after solvent removal by a rotary evaporator. The fluorescence intensity of cells stained by NR is linearly correlated to the gravimetric ratio of cellular lipid.

For instance, the linear correlation between IF values and total lipid extractions for *N. salina* is reported in figure 2.6.

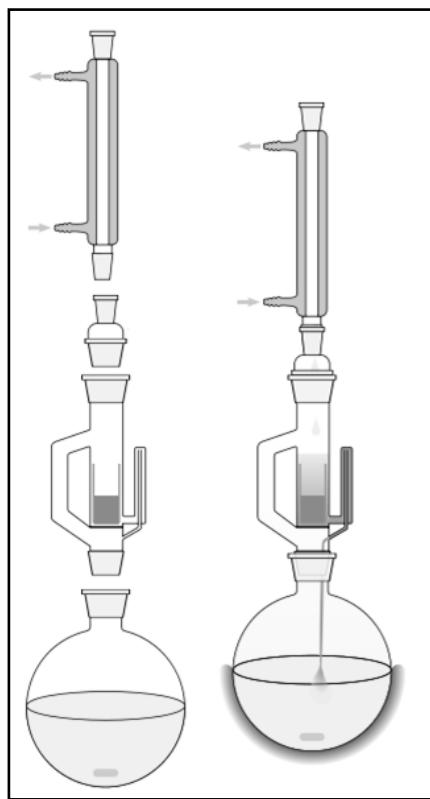


Fig. 2.5: Soxhlet apparatus scheme
(<http://it.wikipedia.org>)

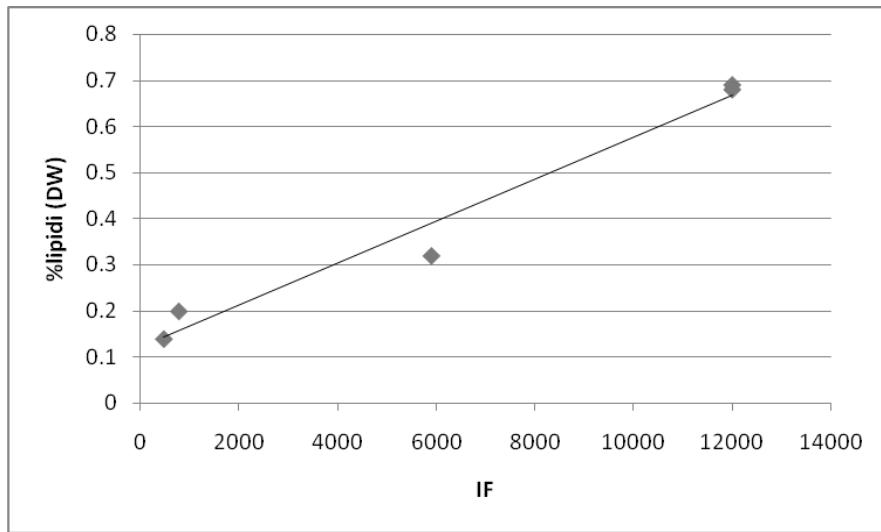


Figure 2.6: Correlation between IF and lipid content for *N. salina*

2.2. Equipments

Growth curves for species screening were carried out in Erlenmeyer flasks, at a final volume of 250 ml. However, as will be reported in further chapters, CO₂ is the most limiting nutrient for algal growth. Thus, a bubbling system was set up, in order to supply a non limiting CO₂ supply. In addition, in order to study the effect of light on growth, a flat panel system was built, and a LED lamp was used, equipped with a light intensity control system.

2.2.1. Experimental apparatus for non limiting CO₂ supply

In order to study the effect of CO₂ concentration on growth, a number of experiments were conducted in a semibatch system, fed by an air flow containing different CO₂ fractions and distributed through a ceramic frit. Thus we set up an experimental apparatus for microalgal growth under CO₂-enriched air bubbling system, shown in figures 2.7 and 2.8). The CO₂ % in air is regulated by two flowmeters. The gases are filtered (microfilter with 0.5 µm of porosity) and bubbled in eight tubular reactors (7 cm diameter and 25 cm high, culture volume of 250 ml), through a metallic frit sparger (figure 2.7). The bottles are contained in a thermostated incubator to avoid culture heating, at 22°C with temperature accuracy of ± 1°C. Artificial illumination, ranging from 5 to 150 µEm⁻²s⁻¹, was carried out with fluorescent lamps. Both mixing of cell suspension and CO₂ transfer are achieved by this bubbling system.

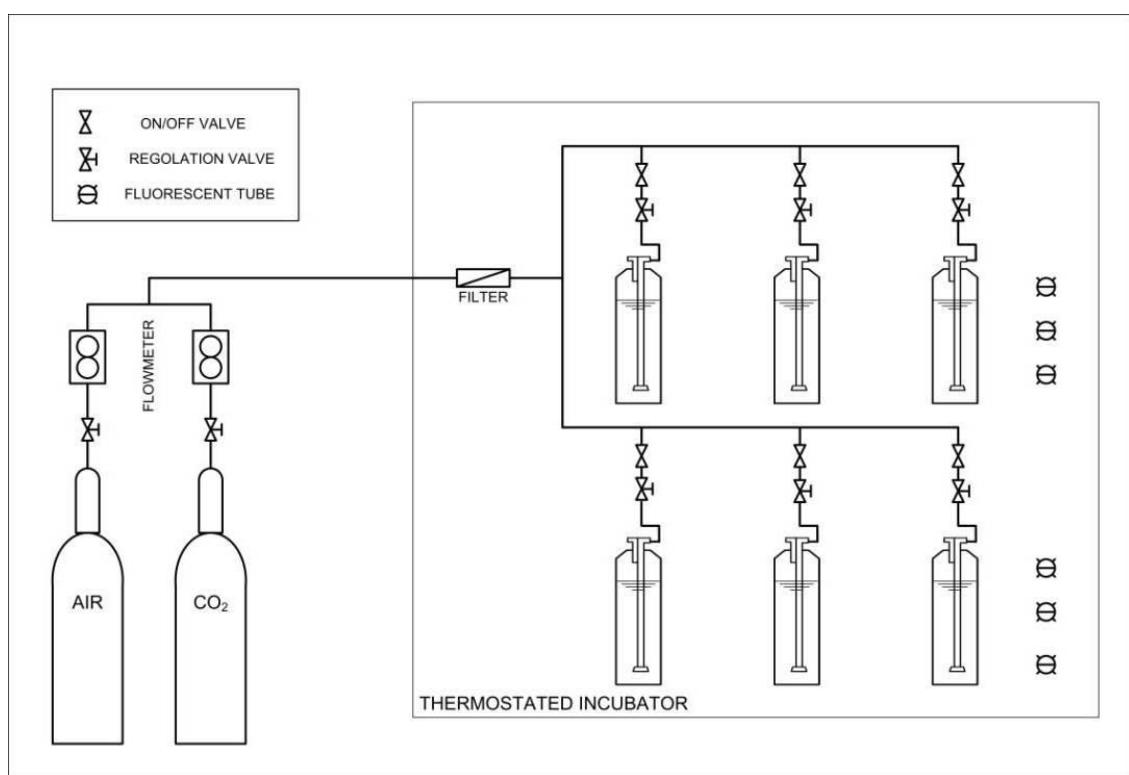


Figure 2.7 Experimental apparatus for microalgae growth under CO₂-enriched air bubbling system.



Figure 2.7 Bubbling tubes for algal cultivation

2.2.2. Flat panels

In order to study the effect of light illumination on growth kinetics, a flat panel system was set up (figure 2.8 – 2.9).

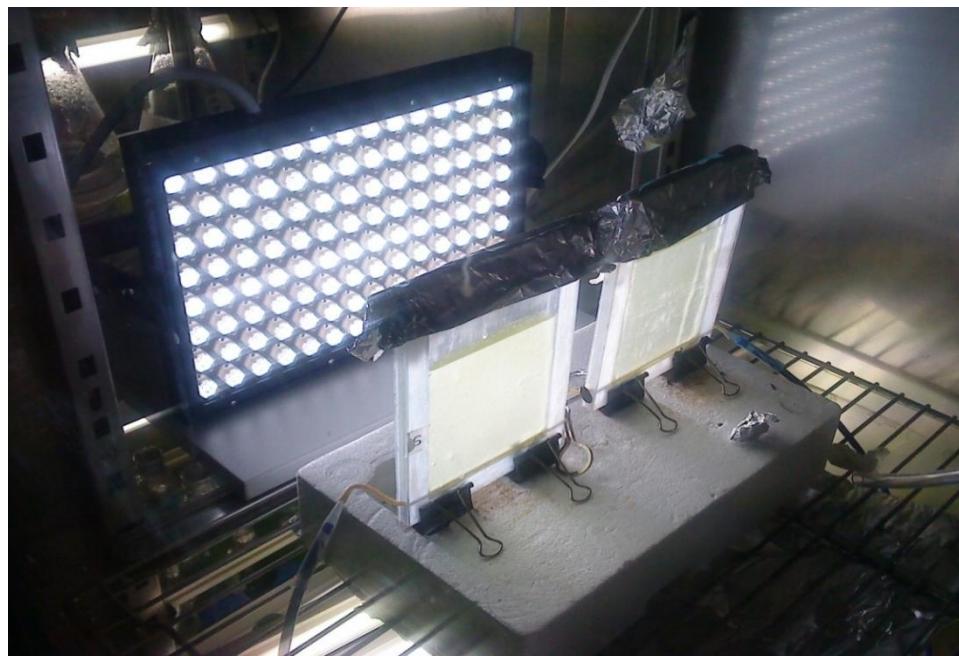


Figure 2.8 Flat panel and LED lamp

In fact, a flat-plate photobioreactor is the most appropriate system for cultivation of photosynthetic microorganisms due to their large illumination surface area. It has been reported that with flat-plate photobioreactors, high photosynthetic efficiencies can be achieved (Zijffers et al., 2010). The flat-plate photobioreactors built and used in this work are made of transparent materials (polycarbonate) for maximum utilization of light energy. The working volume is 150 ml and the culture is mixed by a air-CO₂ flow from a sparger (a silicon tube with holes every 1 cm, obtained by a hypodermic needle of 0.26 mm of diameter) placed in the bottom of the panel. The gas flow also supply a non limiting CO₂ content to the culture. The air entering the reactor is regulated using suitable valves and flowmeters.

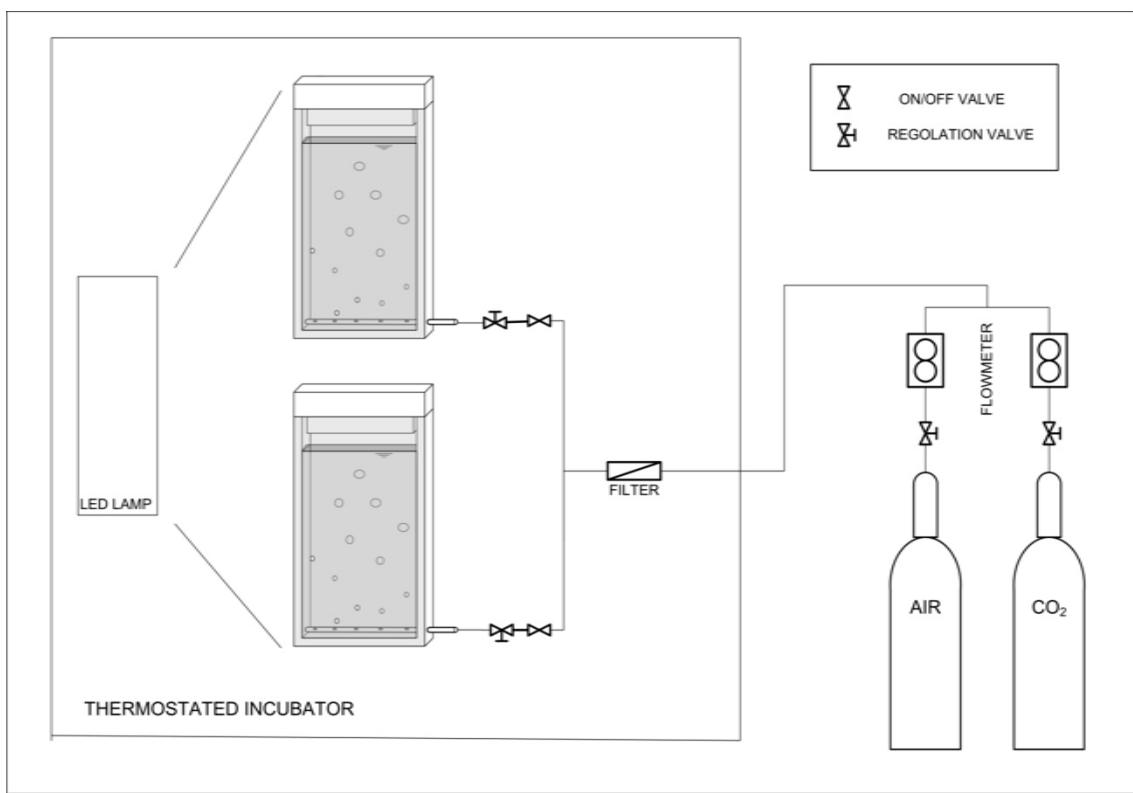


Figure 2.9 Experimental apparatus for measuring the effect of light on growth under CO₂-enriched air bubbling system.

Light is provided by a light emitting diodes (LEDs) lamp (Photon Systems Instruments spol. s.r.o., Czech Republic) (Figure 2.9). LED Light Sources are arrays of high-performance LEDs that are easily controllable in timing - from microseconds to hours - and intensity - from 1 % to 100 % of total output. The size of the light panel is 20x30 cm, and the spectrum of light is natural white. The array is supplemented with a Light Controller that can operate in multiple regimes: flash, continuous light, harmonically modulated light, or user-defined modulation. The Light Controller offers the possibility to create user-defined protocols with light/dark phases and precise control over the light mode, intensity and timing.

2.3. Experimental results of screening

2.3.1. Screening of species

We will discuss first the growth kinetics of the microalgal species, selected from the literature, in order to define the ones that could be potentially used for large-scale production. Results are summarized in table 2.3 and figures 2.10 -2.11 - 2.12. All species were initially grown in Erlenmeyer flasks under conditions according to the literature, to determine the best one with respect to growth parameters.

We found that:

- Under laboratory conditions at temperature of 24°C, *B. braunii* (figure 2.10A) shows a long lag phase. The maximum growth rate was 0.30 days^{-1} , which corresponds to a doubling time of 2.31 days. This species reaches the stationary phase at concentration of 0.52 g/L DW.
- *C. reinhardtii* (figure 2.10B), at 24°C, shows a rather slow growth rate ($\mu=0.13 \text{ d}^{-1}$), corresponding to a doubling time of 5.33 days, with a concentration of 0.74 g/L DW at stationary phase.
- *C. vulgaris* (figure 2.11A) showed the slowest growth kinetics, with a specific growth rate of 0.056 days^{-1} and a doubling time of 12.37 days.
- The species which grew with the higher kinetic constant was *N. limnetica* (figure 2.11B), with a specific growth rate of 0.49 days^{-1} , corresponding to a doubling time of 1.41 days, but it didn't reach high concentration of biomass, that was only 0.12 g/L DW, with a cells concentration of 8 millions of cells per ml.
- *S. quadricauda* (Figure 2.12A) showed a growth rate of 0.18 d^{-1} , corresponding to a doubling time of 3.85 days. The maximum concentration in stationary phase was 0.42 g/L DW.
- *N. salina* (Figure 2.12B), the marine species, was the fastest growing one, showing the best combination of specific growth rate (0.497 d^{-1} , corresponding to a doubling time of 1.39 d), and maximum biomass concentration in stationary phase (0.72 g/L DW).

All growth data are also reported in table 2.3.

Table 2.3. Specific growth rate (μ) of microalgae screened in Erlenmeyer flasks. Maximum concentration data refer to the biomass amount obtained in stationary growth phase.

Species	$\mu (\text{d}^{-1})$	Max. biomass conc. (g/L)
<i>B. braunii</i>	0.30	0.52
<i>C. reinhardtii</i>	0.13	0.74
<i>C. vulgaris</i>	0.056	0.74
<i>N. limnetica</i>	0.49	0.12
<i>N. salina</i>	0.50	0.72
<i>S. quadricauda</i>	0.18	0.42

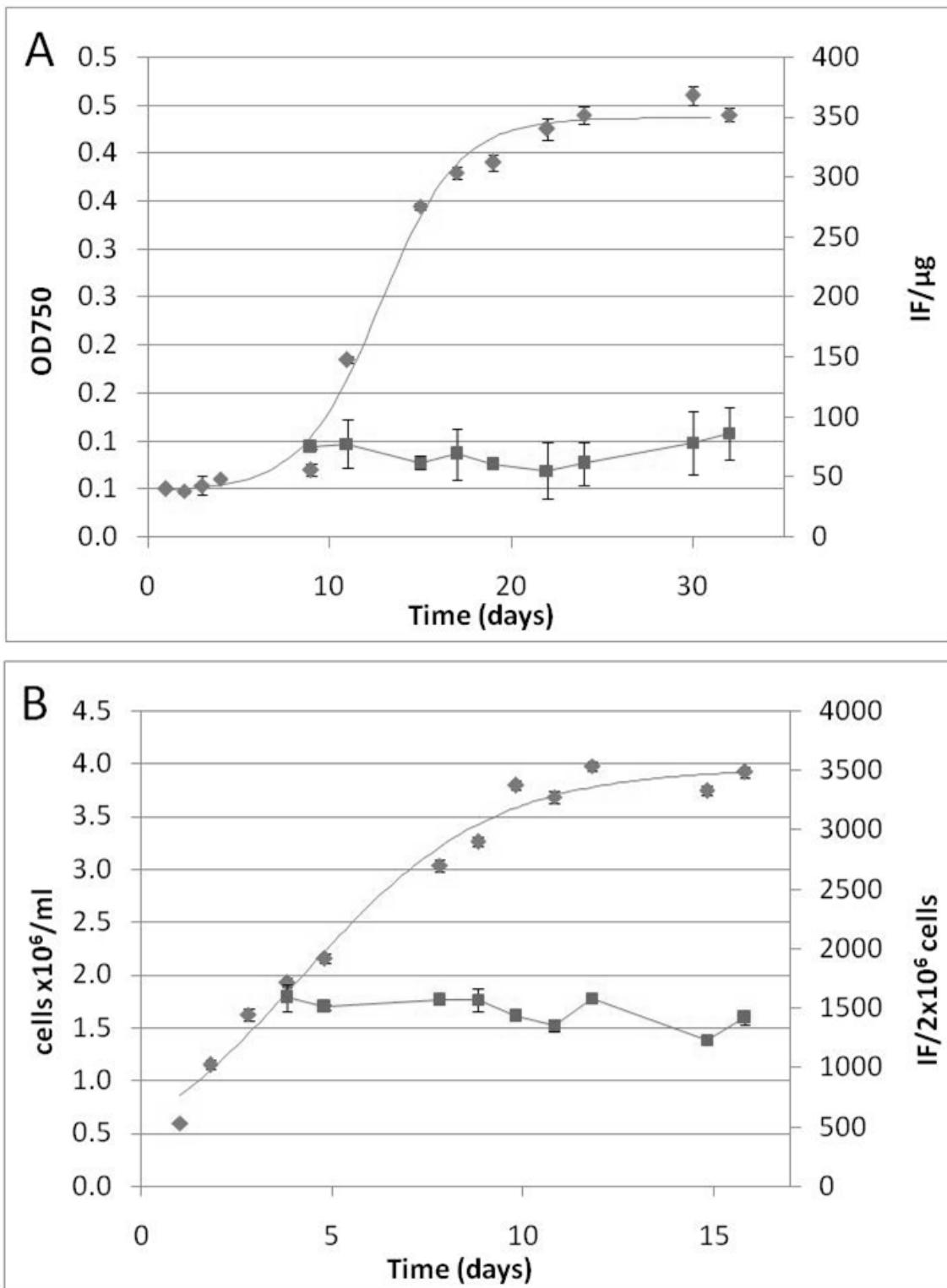


Figure 2.10 Growth curve (◆) and IF of Nile Red staining (■) of *B. braunii* (A) and *C. reinhardtii* (B), under autotrophic conditions and minimum media composition. These runs were performed in Erlenmeyer flasks at 24°C.

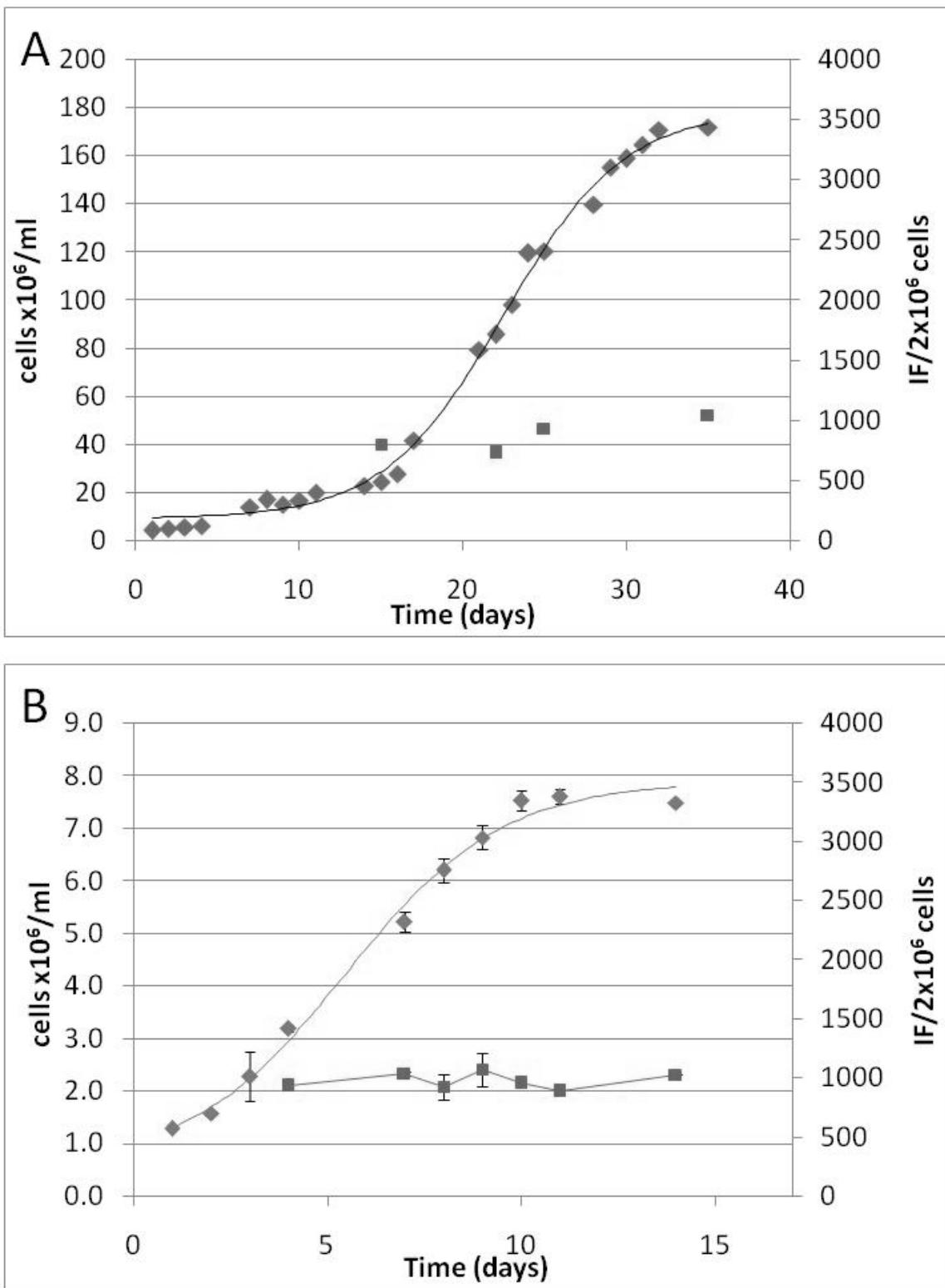


Figure 2.11 Growth curve (♦) and IF of Nile Red staining (■) of *C. vulgaris* (A) and *N. limnetica* (B), under autotrophic conditions and minimum media composition. These runs were performed in Erlenmeyer flasks at 24°C.

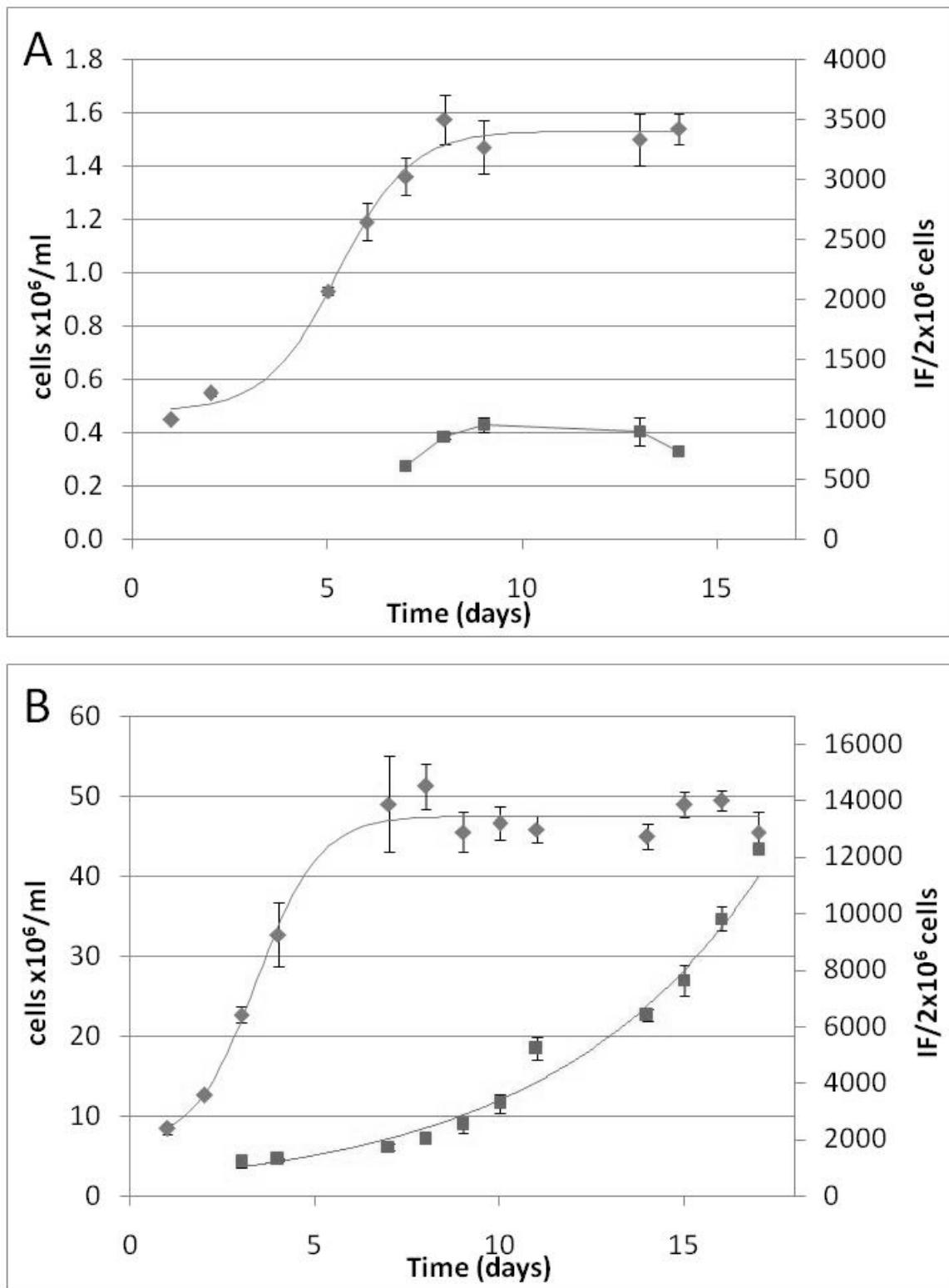


Figure 2.12 Growth curve (\blacklozenge) and IF of Nile Red staining (\blacksquare) of *S. quadricauda* (A) and *N. salina* (B), under autotrophic conditions and minimum media composition. These runs were performed in Erlenmeyer flasks at 24°C.

2.3.2. Lipid content As shown in figures 2.10, 2.11 and 2.12, lipid content of *B. braunii* remained constant during growth, with a fluorescence intensity about 70 per µg of DW biomass, corresponding to about 30% ratio of lipids.

Also for *C. reinhardtii*, *N. limnetica*, *C. vulgaris* and *S. quadricauda*, lipid content remained constant during growth. *C. reinhardtii* showed a Nile Red-staining fluorescence intensity of about 1500 (for 2 millions of cells), corresponding to 20% DW ratio of lipids, in agreement with data reported by (Spolaore et al., 2006), while for *C. vulgaris* it was about 15% DW. *S. quadricauda* accumulated a larger amount of lipids, corresponding to 30% DW. Lipid content of *N. limnetica* was constant during growth, with a fluorescence intensity about 1000 for 2 millions of cells, but the amount of biomass was not enough to perform Soxhlet extraction.

Table 2.4. Maximum lipid concentration observed for each species

Species	Max. lipid conc. (%DW)
<i>S. quadricauda</i>	30±0.5
<i>B. braunii</i>	31±1
<i>C. vulgaris</i>	15±0.5
<i>N. limnetica</i>	--
<i>C. reinhardtii</i>	20±0.2
<i>N. salina</i>	68.5±0.6

As shown in figure 2.12B and table 2.4 *N. salina* emerged as a very interesting species also thanks of its ability to accumulate large amount of lipids, up to about 69% DW. The cellular lipid content changed depending on the growth phase, with the cells sampled in the stationary phase showing a much higher content with respect to those sampled in logarithmic phase, as shown by the increased fluorescence of Nile Red stained cells. Indeed the lipids content increased continuously during the stationary phase reaching a final 69% DW, as reported above. This is likely due to the accumulation of lipids as storage products when growth is limited by the low nitrate concentration in culture media, which becomes limiting in stationary phase (Boussiba et al., 1987).

Interestingly, while no significant lipid accumulation was observed for freshwater species, lipid content increased in the stationary phase in the case of *N. salina*. It is noteworthy that the main difference in the culture media of marine and freshwater species was the nitrate concentration, which was higher for freshwater culture media. In fact, nitrogen is generally far more abundant in freshwater than in seawater (McClintock & Baker, 2001).

There are evidences that nitrogen limitation is responsible of lipid accumulation in microalgae (Illman et al, 2000). Thus, it is possible that the increased lipid content in *N. salina* is caused by

low nitrate concentration in culture media, which becomes limiting in stationary phase. Nevertheless, although also in other species the lipids can be increased by culture conditions optimization, we have decided to focus our further work on *N. salina*, thanks also to its high growth rate.

2.4. Conclusions

In this chapter the set up and the optimization of materials and methods utilized in the thesis were summarized, with particular attention to the techniques for lipid determination. Furthermore, the set up of a semibatch experimental apparatus to non limiting CO₂ supply is reported.

In this chapter experimental results of screening of the species are reported: *Botryococcus braunii* 807.1, *Chlamydomonas reinhardtii* WT8B+, *Chlorella vulgaris* emerson3, *Scenedesmus quadricauda*, *Nannochloropsis limnetica* and *Nannochloropsis salina* 40.85 were cultivated under autotrophic conditions. *N. salina* was selected for its biomass concentration (0.74 g/L DW), growth kinetic parameters ($\mu = 0.497 \text{ days}^{-1}$) and lipid content (69%).

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CHAPTER 3

PHOTOBIOREACTORS FOR MICROALGAL GROWTH AND OIL PRODUCTION WITH *Nannochloropsis salina**

FROM LAB-SCALE EXPERIMENTS TO LARGE-SCALE DESIGN

This chapter reports new experimental data of microalgae growth and lipid production under autotrophic conditions for the species *Nannochloropsis salina*. The effect of relevant operating variables is addressed and discussed, and some suggestions to better understand the process behavior are given with respect to lipid content maximization, carbon dioxide and nitrogen supply, and illumination conditions. The data obtained are finalized to the design of an environmentally and economically sustainable photobioreactor in view of achieving industrial photosynthetic biomass and natural oil production from large scale microalgae cultivation.

3.1. Introduction

The interest in microalgae cultivation is currently very high because microalgal oil, among other uses, could represent an alternative to complement and eventually replace fossil fuels in the years to come.

Research concerning lipid production from microalgae was stimulated in the past three decades by the increasing shortage of crude oil. Natural oil from microalgae seems to be the only renewable biofuel with the potential to completely displace petroleum-derived transport fuels because, at least in principle, it can be employed for the production of biodiesel in an economically effective and environmentally sustainable manner (Chisti, 2007). Although this point has been recently questioned by a life cycle analysis (LCA) (Clarens et al., 2010), the unavailability of industrial plant commercial data currently hinders any adequate LCA study, and more research efforts are needed not only to show the process feasibility at the industrial scale, but also to provide further experimental and theoretical support to the implied technology.

Microalgae are a highly diverse group of unicellular photosynthetic organisms comprising eukaryotes and prokaryotic cyanobacteria, that can grow at a much faster rate than plants thanks to their simple structure. Unlike the case of first-generation biodiesel, the extensive cultivation of microalgae does not compete for agricultural land with food crops, since they can be grown on marginal land or in aquatic systems. However, in order to become economically feasible, the cultivation of microalgae for biofuel production requires high biomass and lipid productivity per area and minimum plant investment and operating costs.

The two options for massive production of microalgae are open systems, such as raceway ponds, and closed photobioreactors. Open cultures are inexpensive but bring about important drawbacks, including lower long-term productivity due to limited exposition to light, complex carbon management and large susceptibility to contamination (Wahal and Viamajala, 2010). In the case of photobioreactors, initial capital investments are certainly more demanding, but they can provide higher overall productivity thanks to better contaminant management and improved utilization of photosynthetically active radiation, carbon dioxide and other nutrients. The design of closed systems must be carefully optimized for each individual microalgal strains, according to its specific physiological and growth characteristics. Apparently, major technical and economic challenges still prevent the selection of an optimal reactor type at the commercial scale (Kunjapur and Eldridge, 2010).

Several researchers worldwide claim to possess technologies for the commercial production of biodiesel from microalgae, and many new companies have been recently developed(Singh and Gu, 2010; Zijffers et al., 2010; Grobbelaar, 2010). However, a number of technical challenges remain unsolved, including questions concerning large-scale microalgae recovery and oil separation processes. For these reasons, in spite of quite large scientific, technological and commercial interest, no industrial plants finalized to produce oil from microalgae are operated in the world (Singh and Gu, 2010): algae cultivation systems work well at the laboratory and small pilot/demonstration levels, but the process feasibility has not been demonstrated for large scale production yet.

One of the problems is that the best microalgae productivity achieved in pilot units does not exceed 10% of the maximum theoretical limit (Rodolfi et al., 2009). In order to increase this value, it is first of all essential to understand the fundamental phenomena involved. At the biological/biochemical level, research is currently focused in increasing the lipid content and quality of microalgal strains (Chisti, 2007) also through genetic modifications (Courchesne et al., 2009). From the chemical engineering standpoint, parameters such as temperature, light regime, nutrient feed system, heat and mass transfer have to be finely tuned with substantial improvement of the photobioreactor set-up and operation. Of particular importance is also ensuring adequate supply of nutrients to avoid they can be growth-rate limiting factors.

Phototrophic microalgal production requires light, carbon dioxide, water and inorganic nutrients. Light is providing all energy for biomass accumulation and its absorption and conversion efficiency must be maximized in microalgal mass culture systems (Chisti, 2008) (Carvalho et al., 2011). Another main issue to be addressed is carbon supply. Flue gases from power and chemical plants could represent an inexpensive CO₂ source (Chisti, 2007), but its concentration must be optimized avoiding both growth limitation by low CO₂ supply and also growth inhibition due to excess acidification (Ruiz-Marin et al, 2010). Besides carbon, nitrogen and phosphorus are the most important nutrients, for which a promising approach is using mineral salts contained in wastewaters from treatment plants (ammonium, nitrates and phosphates), thereby contributing also to the solution of water eutrophication problems (Ruiz-Marin et al, 2010; Chinnasamy et al., 2010). The combination of biofuel production from algae with CO₂ sequestration and wastewater bioremediation could result in an eco-friendly process and provide economical advantages to improve the still uneconomical large-scale second generation biofuel production system (Sheehan et al., 1998).

Although metabolic regulation in microalgae is still poorly understood, it is well known that their molecular composition is affected by nutrient availability in the growth medium. In particular, cellular lipids content can be regulated by nutrient availability during growth (Rodolfi et al., 2009; Converti et al., 2009). Interestingly, nitrogen deficiency was shown to enhance lipid accumulation in different algal species (Oswald, 1988; Illman, et al., 2000). However, a strong increase in lipids accumulated comes at the expense of a reduction in biomass growth, thus reducing overall productivity (Converti et al., 2009). In order to maximize both biomass growth and lipid synthesis, a two-phase strategy was successfully experimented: a first step with nutrient sufficient and high biomass production was followed by a step of nutrient depletion with lipids accumulation (Rodolfi et al., 2009).

The objective of this chapter is to study the effect of several parameters on microalgae growth and lipids accumulation, in order to better understand this process, and to give a contribution towards its industrialization and large-scale application. To this aim we focused on the marine species *N. salina* for its high biomass productivity and ability to accumulate large amounts of lipids (Boussiba et al., 1987). We evaluated growth performances and lipid content in this species in various culture conditions. In particular we focused on the effect of nitrogen content, CO₂ supply and concentration and light/dark cycles. Finally, based on our experimental results, we summarized the parameters which could be more relevant in the design of large-scale photobioreactors for *N. salina* production.

3.2. Materials and methods

The microalgae species used was *Nannochloropsis salina* 40.85, cultivated in flask with the medium described in paragraph 2.1.1. For the experiments with different sources or

concentration of nitrogen, the basic media was modified by changing nitrogen content and concentration (0.71 g/L of NH_4NO_3 (Sigma-Aldrich) and 1.2 g/L of $(\text{NH}_4)_2\text{SO}_4$ (Sigma-Aldrich) added to basic media for source of nitrogen experiments; 1.5 or 2 g/L of NaNO_3 (Sigma-Aldrich) added to basic media for optimization of growth experiments). In order to study the effect of CO_2 concentration on growth, a number of experiments were conducted in the semibatch system, fed by an air flow containing different CO_2 fractions and distributed through a ceramic frit (see Figure 2.7, paragraph 2.2.2). For the nitrogen-starvation experiments, cultures in the early stationary phase were centrifuged at 5000 g and re-suspended in nitrogen depleted medium (0.075g/L NaNO_3). Cell concentration, OD and lipid content were measured as described in paragraph 2.1.

3.3. Results and discussion

N. salina confirmed to be a fast growing species, if compared with other species screened, showing a good combination of specific growth rate (0.497 d^{-1} , corresponding to a doubling time of 1.39 d), and maximum biomass concentration in stationary phase (0.72 g/L DW), also because of its ability to accumulate large amount of lipids, up to about 69% DW, as reported in section 2.4.

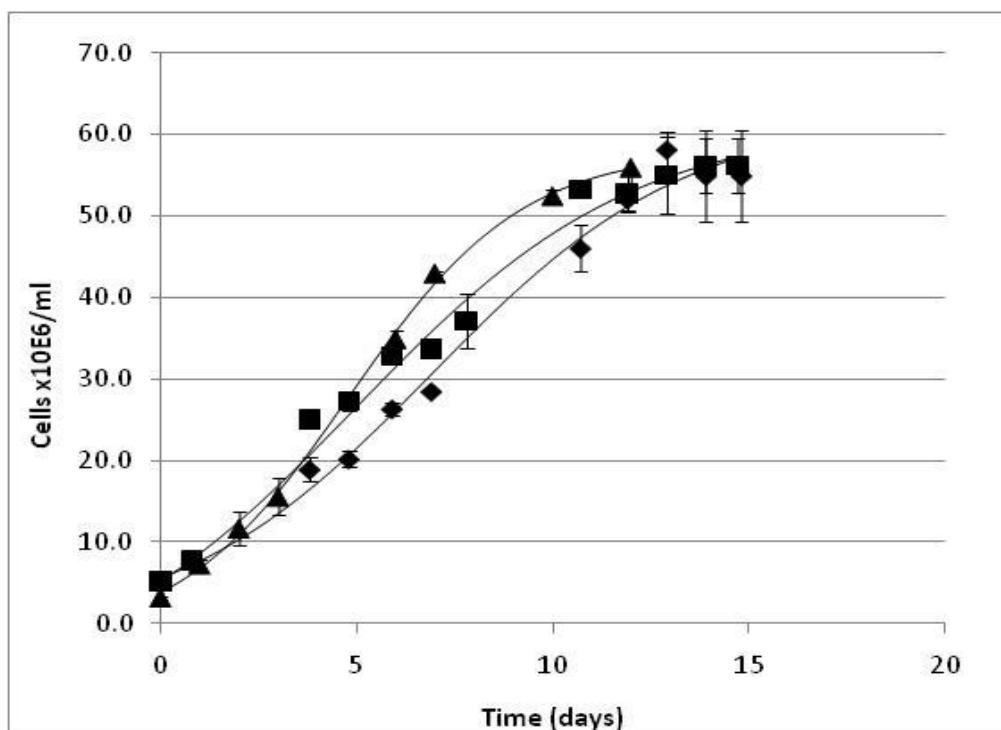


Figure 3.1. Growth curves of *N. salina*, under autotrophic conditions with different sources of nitrogen. Different concentrations of salts were employed to reach the same nitrogen content: the added concentration were respectively: (■) 1.5 g/L NaNO_3 ; (♦) 0.71g/L NH_4NO_3 ; (▲) 1.2 g/L $(\text{NH}_4)_2\text{SO}_4$. In all cases nitrogen concentration was about 18 mM. These runs were performed in Erlenmeyer flasks.

3.3.1. Impact of different nitrogen sources

Considering the seminal influence of nitrogen on lipids productivity, the effect of different nitrogen sources was investigated. In culture media of microalgae species, nitrogen is commonly supplied as nitrate (NO_3^-), although ammonia (NH_4^+) and urea are also used for some species (Kaplan et al., 1986).

In order to verify its ability of consuming different nitrogen sources, *N. salina* was cultured in the presence of NaNO_3 , NH_4NO_3 , or $(\text{NH}_4)_2\text{SO}_4$ at constant molar concentration of nitrogen. In all the conditions examined, growth curves are similar and show the same time course and biomass accumulation (see figure 3.1); lipid accumulation is also unaffected by the use of different nitrogenated compound (data not reported). We conclude that *N. salina* can use both ammonia and nitrate as nitrogen source, an important ability in the perspective of developing of an integrated process for oil production and waste-water treatment.

3.3.2. Effect of light and photoperiod conditions

In photosynthetic cultures, the light energy is used by the cells either for maintenance processes or for formation of new biomass (Pirt, 1986). Consequently, the biomass productivity and the cell growth rate are directly linked to the light energy available, which varies from day to night.

Table 3.1. Specific growth rate (μ) of *N. salina* under continuous illumination and dark-light cycle. Maximum concentration data refer to the biomass amount obtained in stationary phase.

Light regime	μ (days $^{-1}$)	Max. biomass conc. (g/L)
Continuous illumination	0.42	0.77±0.01
Dark-light cycle	0.26	0.54±0.01

We characterized the growth kinetics and biomass accumulation of *N. salina* under continuous illumination and 12:12 (night:day) cycles. Data in Table 3.1 and Figure 3.2 show that the specific growth rate of *N. salina* under light/dark cycle is 38% lower with respect to continuous illumination and that the biomass concentration in stationary phase is about 30% less. The reduction in biomass accumulation can be taken as a rough estimation of the energy used for cellular maintenance during the dark periods, which is crucial to predict the behavior of a large-scale photobioreactor. However, it should be pointed out that, since in 12:12 (night:day) cycle only 50% of the light energy was provided, the reduction means that cells are more efficient during the day than under continuous light.

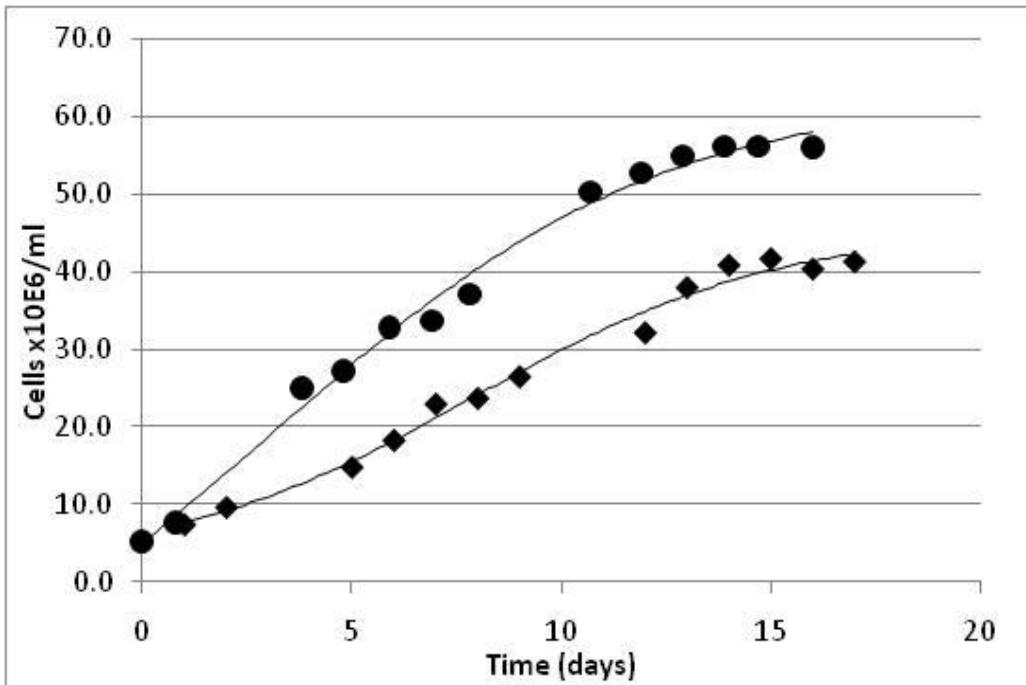


Figure 3.2. Growth curves of *N. salina*, under continuous illumination (●) and under (12:12) dark-light cycle (◆). These runs were performed in Erlenmeyer flasks.

3.3.3. Effect of CO₂ transfer and nitrogen supply on growth

Current CO₂ transport technology in mass algal culture systems suffers the shortcomings of inefficient CO₂ transfer across the gas–liquid interface and loss of valuable CO₂ in the exhaust air (Heussler et al., 1978; Richmond and Becker, 1986). The CO₂ concentration in water depends both on thermodynamics and mass transfer. Given a CO₂ partial pressure in the bubbling gas phase, its equilibrium composition in water can be calculated by the Henry law:

$$y = \frac{H \cdot x}{P} \quad (3.1)$$

where x , y are the CO₂ mole fractions in the liquid and gas phases, respectively, P is the pressure and H is the CO₂ Henry constant, which is a function of temperature T according to:

$$H = \exp \left[A + \frac{B}{T} + C \cdot \ln(T) + D \cdot T \right] \quad (3.2)$$

The values of parameters A , B , C , D were taken from (Kundu and Bandyopadhyay, 2006) and are reported in Table 3.2. According to Eqs. 1-2, it can be calculated that the CO₂ equilibrium concentration in water is about 0.56 mg/L at atmospheric CO₂ concentration, and about 80 mg/L with 5% of CO₂ in the gas phase.

Table 3.2. Parameters to calculate the Henry constant as a function of temperature.

A	B	C	D
170.7126	-8477.711	-21.95743	0.005781

The CO₂ mass transfer from the gas-liquid interface to the liquid bulk ($W_{CO_2,TR}$, in kmol/s m³) can be expressed by:

$$W_{CO_2,TR} = (k_L a)_{CO_2} (c_{CO_2}^* - c_{CO_2}) \quad (3.3)$$

where $c_{CO_2}^*$ and c_{CO_2} (kmol/L) are the CO₂ concentrations in equilibrium with the gas phase and in the liquid bulk, respectively, and $(k_L a)_{CO_2}$ is the CO₂ volumetric mass transfer coefficient (1/s). The $k_L a$ value can be estimated by experimental correlations obtained in bubbled column and airlift reactors, as proposed in the literature (Camacho Rubio et al., 1999; Mazzucca Sobczuk et al., 2000). As a reasonable and precautionary value to calculate the minimum gas phase concentration we assumed $k_L a = 0.002 \text{ s}^{-1}$ (Chisti and Jauregui-Haza, 2002).

Table 3.3. Elemental chemical analysis of *N.salina*.

Element	% p/p
C	59.51
H	9.29
N	6.46
O	24.72

Elemental chemical analyses, performed as reported in paragraph 2.1, showed (Table 3.3) that algal biomass consists of 30% to 60% carbon, suggesting that the production of 1.0 kg of biomass requires 1.5 to 2.0 kg of CO₂ (Mazzucca Sobczuk et al., 2000). Assuming a biomass productivity of 1.5 kg/(m³ d), microalgae are able to fix 3 kg/(m³ d) of CO₂ as biomass and this amount must be transported according to eq. 3, resulting in a minimum CO₂ mole fraction in the gas phase of around 3% to avoid any limiting effect caused by CO₂ transfer.

We also checked in the semibatch apparatus that CO₂ mass transfer was limiting for algae growth. As reported in Figure 3.3A, with 5% of CO₂ the culture reached a cells concentration in stationary phase of about 64 millions of cells/mL, higher than with atmospheric air (about 48 millions of cells/mL). Furthermore growth rate constant was significantly higher as well (0.590 d⁻¹ versus 0.497 d⁻¹) confirming that, with atmospheric air, the CO₂ supply to the cultures is limiting. Therefore, all the further semibatch experiments reported were carried out with air containing 5% of CO₂.

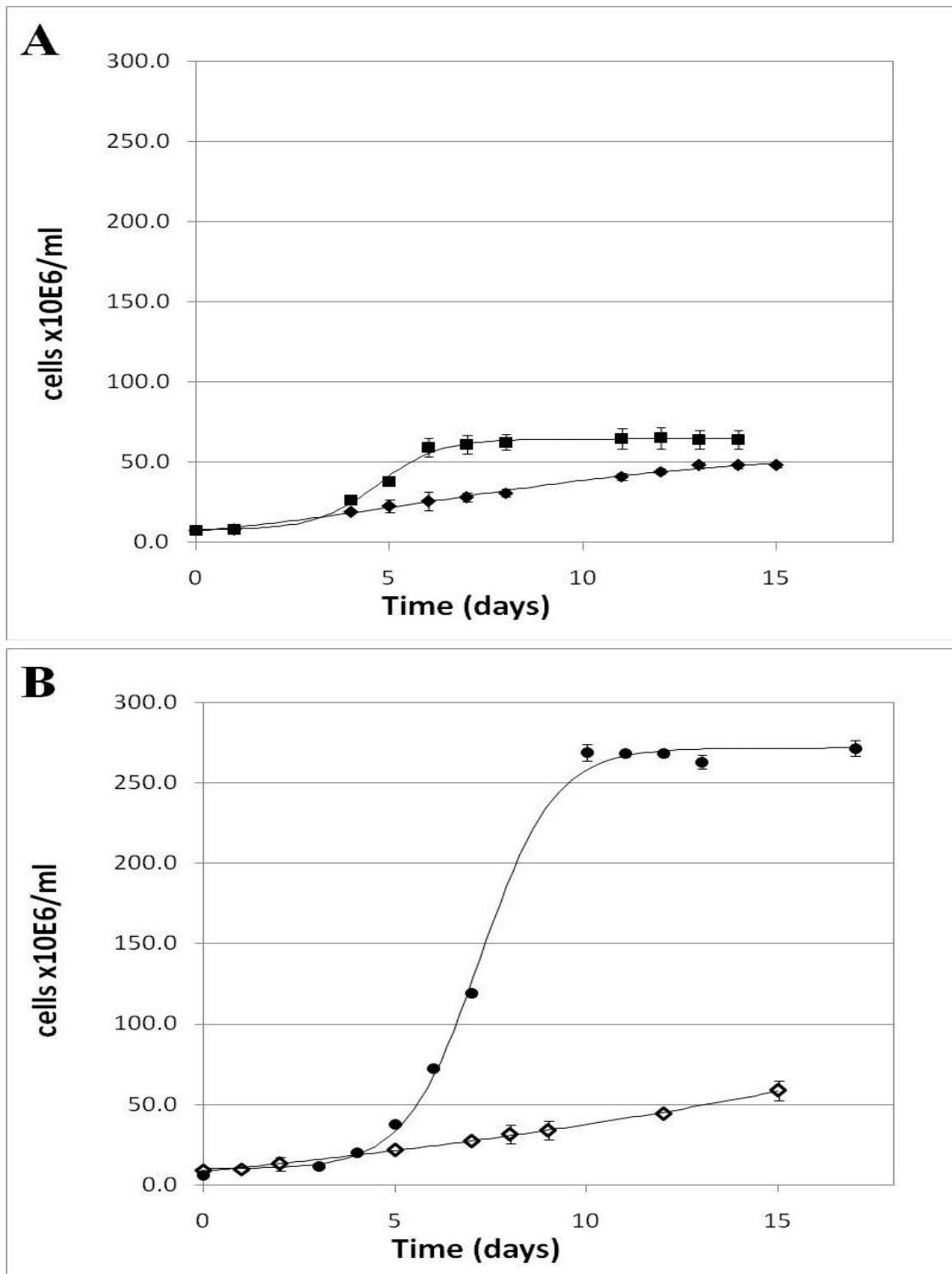


Figure 3.3. Effect of CO₂ (5% v/v in air) on growth curve (■) of *N. salina* (Fig.3.3 A), compared to atmospheric air-bubbled culture (◆). Fig.3.3B: effect of additional NaNO₃ (1.5g/L) on cell growth of *N. salina*, with atmospheric air (○) and 5% CO₂-enriched air (●).

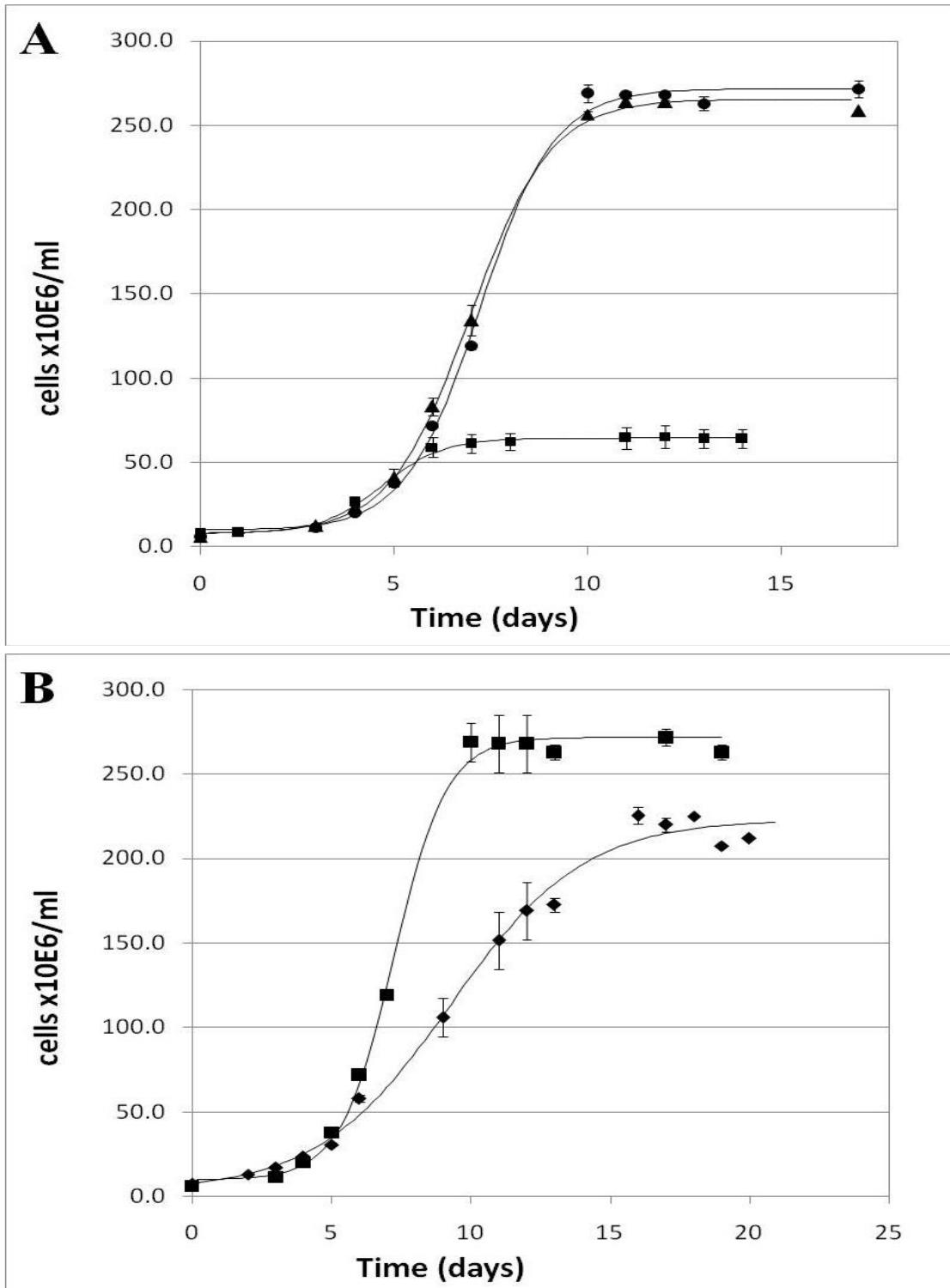


Figure 3.4. In fig. 3.4A the effect of NaNO_3 concentration on *N. salina* cell growth, with 5% CO_2 -enriched air, is evidenced: 0.075 g/L NaNO_3 (■); 1.5 g/L NaNO_3 (●); 2 g/L NaNO_3 (▲). In fig. 3.4B, growth curves with different 5% CO_2 -enriched air flow rates: 1 L/h (■) and 0.25 L/h (◆).

Nitrogen availability played a even larger effect than CO₂ on algae growth. In fact, using a nitrogen enriched medium, a specific growth rate of 0.599 days⁻¹ and maximum cells concentration of about 270 millions of cells/mL (corresponding to 2.87g/L DW) were achieved (Figure 3.3B). No further increase of biomass was obtained with NaNO₃ concentration higher than 1.5g/L, showing that this concentration is already in excess in our experimental conditions (see Figure 3.4A). Interestingly, these results were achieved only if CO₂ was provided in excess and the addition of sodium nitrate with atmospheric CO₂ was totally inefficient (figure 3.4B), while enrichment of both nutrients increased the biomass concentration by more than a factor of 4 (figure 3.3A).

Of course, the CO₂ concentration in the liquid may fall below the limiting value also because of insufficient supply rate, and for this reason we checked the effects of gas flow rate on growth kinetics. As reported in figure 3.4B, when the flow is 0.25L/h, growth rate is reduced to about 0.410d⁻¹ compared to the 0.590 d⁻¹ measured in experiments with 1L/h. Also the maximum biomass concentration reached at stationary phase is 2.69g/L instead of 2.92 g/L, confirming that, in our apparatus, 0.25L/h are not sufficient for supporting algae maximal growth.

When CO₂ is limiting, reducing power generated by photosynthetic chain cannot be completely exploited by the Calvin-Benson Cycle leading to thermal dissipation of the absorbed light energy. Moreover, over-reduction of PSII electron acceptors leads to production of activated oxygen species with permanent or slowly reversible oxidative damage to the photosynthetic apparatus. Therefore, a culture growth limited by the CO₂ concentration not only decreases the growth rate, but also impairs the global mass production due to lower photosynthetic activity. This is not the case when nitrogen (NO₃) is limiting: in these conditions nitrogenated compounds such as nucleotides and aminoacids cannot be duly synthesized, but the photosynthetic cycle may well go to completion with accumulation of carbohydrates and lipids.

3.3.4. Effect of nitrogen on lipid accumulation

When nitrogen is present in excess, the cellular lipid content is constant during the growth curve, as monitored from the fluorescence intensity upon staining with Nile Red (Figure 3.5A).

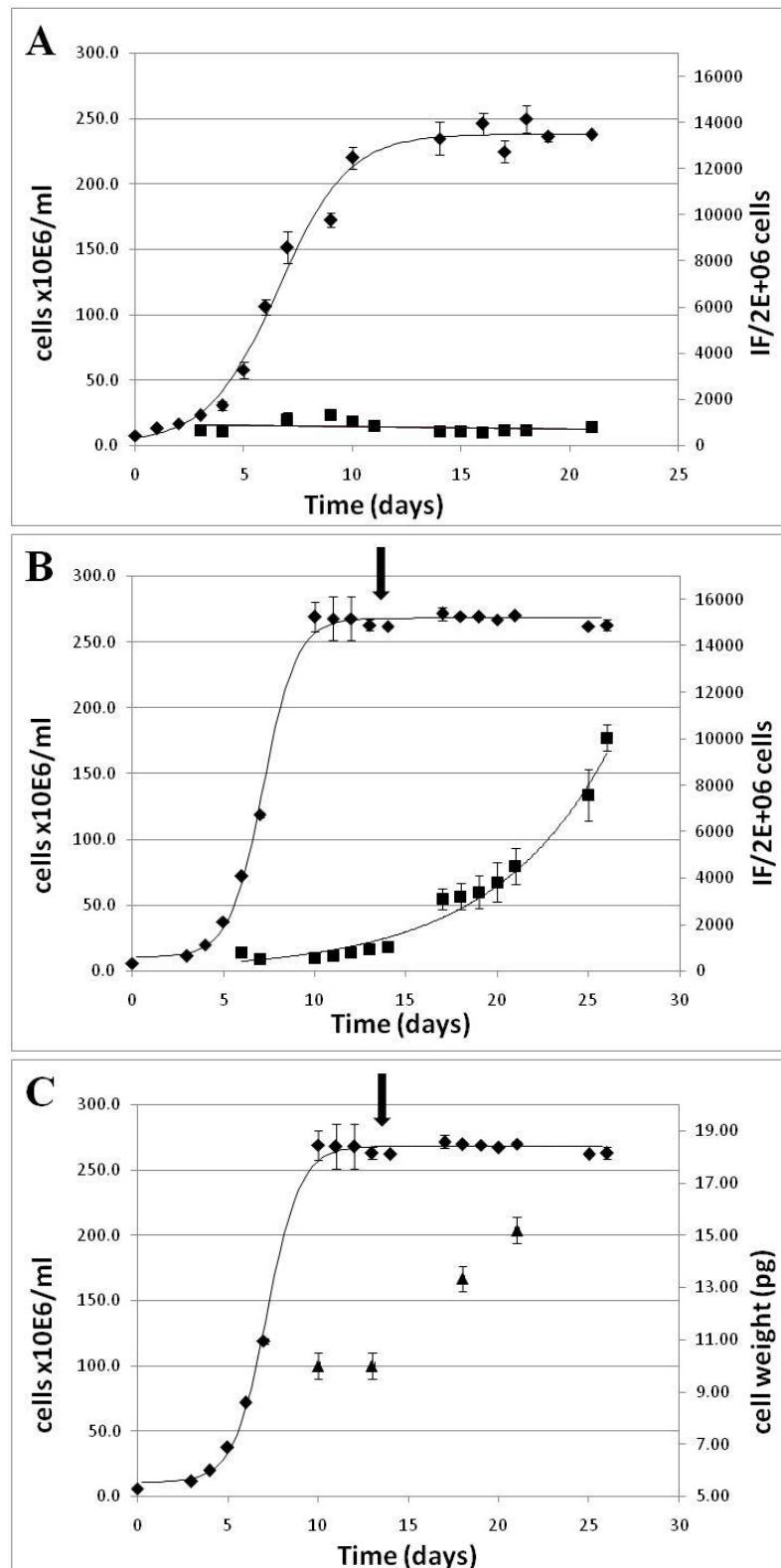


Figure 3.5. Growth curves (◆) and IF of Nile Red staining (■) of *N. salina*, under autotrophic conditions and 1.5g/L of NaNO_3 (Fig. 3.5A). In fig. 3.5B the effect of N-limitation on growth (◆) and IF (■) is reported: the arrow indicates the centrifugation and resuspension of culture in nitrogen depleted medium. In fig. 3.5C the increase of weight per cell (▲) during time is reported. These runs were performed in the semibatch apparatus.

On the contrary, results previously reported in Figure 2.11B showed a large lipid accumulation in late growth phase which is likely due to nitrogen limitation. To verify this hypothesis, we collected cells by centrifugation in the early stationary phase, and re-inoculated them into N-deficient medium. As shown in Figure 3.5B, the lipid content increased up to a fluorescence intensity of about 11000, which was quantified gravimetrically to correspond to a lipid content of $63 \pm 1\%$ DW. Interestingly in this growth phase we observed a net increase in biomass (up to a concentration of 4.05 g/L DW), while the cells number remained constant (Figure 3.5C), indicating that, under the selected conditions, *N. salina* continues to fix carbon dioxide which is mainly used for lipid biosynthesis.

In summary, experimental results clearly show that a high concentration of nitrates during the earlier phase of growth stimulates the biomass formation, but *N. salina* accumulates lipids only under nitrogen deficiency, as previously observed by other authors (Rodolfi et al., 2009).

3.3.5. Effect of CO_2 on the pH

Another relevant issue in a large scale photobioreactor is a possible change in pH, due to the dissolution of carbon dioxide in the liquid phase (Livansky, 1990).

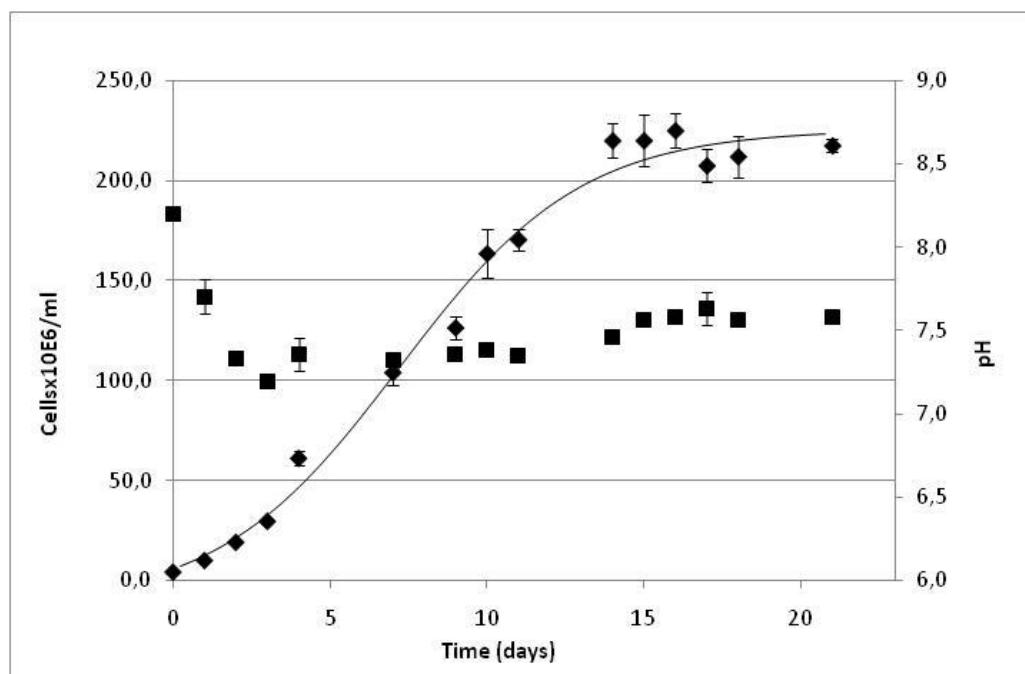


Figure 3.6. Change of pH of culture medium bubbled with CO_2 5% in air, during microalgal growth.

In the presented experiments we stabilized the pH using a buffer system (TRIS-HCl) for the culture media. Despite this, as shown in Figure 3.6, the pH of the culture medium initially decreases from 8 to 7.2 but after 4 days of growth, when the concentration of cells is higher, the pH increases again up to 7.5, due to the greater consumption of carbon dioxide by the cells. These pH changes are negligible, as the values are within the tolerability range of the species, and thus the ratio between buffering power and CO₂ influx is suitable for achieving maximal growth.

3.3.6. Photobioreactor design considerations

The experimental results obtained in this chapter confirm and extend the findings from other authors about the main operating variables governing cell growth and lipid production in a photobioreactor for *N. salina* cultivation. Depending on them, it is interesting to discuss which variables are more relevant when moving from the laboratory scale to a large production unit. The behavior of a large-scale photobioreactor have been addressed and discussed very recently (Kunjapur and Eldridge, 2010; Singh and Gu, 2010; Mata et al., 2010), without providing final directions, so that some further considerations seem appropriate.

A first question is related to the step which may limit algae growth in mass production units: is it carbon dioxide availability, or nutrient availability, or energy supply, or a combination of them?

From our results it may be concluded that both carbon dioxide and nitrogen supplies do not represent limiting factors as far as material balance and mass transport requirements are fulfilled. For an efficient photobioreactor design the CO₂ supply should never be limiting during day-light period, as this condition not only would negatively affect the growth rate but also would damage the photosynthetic apparatus with global loss of biomass production. 1.5 to 2.0 kg of CO₂ are needed per kg of algae produced, while one tenth of this amount is due for nitrogen (Weyer et al., 2010). The CO₂ quantities available from power coal-fired combustion processes are of the order of 15 Gt/y and would be enough to produce the algal oil needed for sustaining the entire world transport fuel demand.

As for nitrogen, animal farms could provide the entire amount required for algal growth (Mata et al., 2010). Similar figures hold for other nutrients (phosphates and trace minerals). In addition, for both CO₂ and nitrogen, their concentrations in available waste streams are compatible with those suitable as a feed of an algal photobioreactor.

Of course, the practical possibility of using residues to grow microalgae is an open issue (Pittman et al., 2011), but preliminary results seems to be promising.

Concerning the process energy duty, in autotrophic conditions it must be provided by solar radiation only. To develop a simple rule-of-thumb calculation, “regular” values of the solar radiation flux (a yearly average of 6000 MJ/m², as it is at 40° latitude) and of the heat of

combustion of dry microalgae (20 MJ/kg) can be assumed. Taking into account that the maximum theoretical photon conversion efficiency is about 12% (Lien and San Pietro 1975), it can be calculated that at best $36 \text{ kg}/(\text{m}^2 \text{ y})$ of algae (DW), i.e. 365 ton per year per hectare, can be produced to fulfill the energy supply constraint. Also in such an ideal condition a photobioreactor autotrophically operated needs huge amounts of light-exposed surface to ensure algae mass production.

On the other hand, in a perfectly mixed reactor where the algae concentration is assumed to be 2.0 g/l, and with an average growth constant of 0.5 d^{-1} the production rate would be $1 \text{ kg}/(\text{m}^3 \text{ d})$, that is $360 \text{ kg}/(\text{m}^3 \text{ y})$. If the maximum production rate value of $36 \text{ kg}/(\text{m}^2 \text{ y})$ is divided by this number, an average depth of 10 cm is obtained for a hypothetical perfectly stirred flat photobioreactor. Thus, under the proposed assumptions, a reactor 10 cm deep would be needed to achieve such a production and there would be no reasons to use a deeper pond since the limiting factor for the process.

Recent experimental results (Zijffers et al., 2010) reported that the maximum theoretically attainable biomass yield can be approached in a flat panel photobioreactor of 1 to 2 cm thickness. Under real operating conditions the solar radiation absorption efficiency is far less than 100%, and also the average growth rate constant may be less than 0.5 d^{-1} , but the final result of a shallow reactor seems to be confirmed, as long as it is able to absorb all the incident light. Therefore, increasing the photobioreactor volume per light exposed area does not seem to give any advantage. In addition, there is no need to adopt complex photobioreactor geometries for large scale applications, as the simplest one, that is a flat and thin panel, is suitable to effectively transform light into biomass in the most simple and inexpensive way.

Some other issues need to be addressed and solved for a proper reactor design. Among them, we quote the improvement of photosynthetic efficiency, the maximization of lipid content, the operation overnight to reduce biomass losses, the reactor contamination to avoid washout, the effect of secondary metabolites, the controls of temperature, pH and mixing in the reactor. These questions have to be addressed to find out an economically sustainable way of achieving microalgae mass production, but the limit of biomass yield per hectare should be always kept in mind. Even though the throughput of around 350 ton/(ha y) of biomass is more than one order of magnitude higher than for any terrestrial crop, and can be obtained with markedly less load of freshwater sources, microalgae cultivation will never allow to industrially produce relevant amounts of biomass without the utilization of huge land surface.

3.4. Final remarks

The development of large-scale photobioreactors for natural oil massive production from microalgae requires a thorough understanding of the phenomena and of the key operating variables involved in biomass growth and lipid formation. This problem was investigated using *N. salina* species as a study model. The kinetics of biomass growth of this species was measured under conditions of non-limiting nutrient supply, as it was shown for carbon dioxide and nitrogen in a novel semi-batch apparatus, accounting for both thermodynamic and mass transfer considerations. The lipid concentration in the algae was maximized by applying nitrogen deprivation. Experimental runs were carried out also under dark/light cycles, to mimic the real situation. The results obtained are necessary, but not sufficient, to design a large-scale unit, for which the energy duty, supply and light absorption of the process must be considered first. It was shown that the limit of biomass yield per unit land used, which depends on light availability rather than on the intrinsic growth rate, may impact on the reactor geometry, which was suggested to be kept as simplest as possible also for economic reasons.

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CHAPTER 4

MIXOTROPHIC GROWTH OF *Chlorella protothecoides* and *Nannochloropsis salina* UNDER NON LIMITING CO₂ SUPPLY*

The aim of this chapter is to verify the possibility of exploiting mixotrophy to support algal growth overnight or in dark zones of a photobioreactor. Two species with high biomass productivity were selected, one marine, *Nannochloropsis salina*, and another one growing in freshwater, *Chlorella protothecoides*. Different organic substrates were employed as carbon source (acetate, ethanol and glycerol), chosen for their availability at industrial scale. The comparison of growth kinetic parameters, maximal biomass concentration and lipid accumulation under autotrophic and mixotrophic conditions showed that both *N. salina* and *C. protothecoides* display higher productivity in the presence of glycerol, which thus represents a promising organic substrate to support algal growth at industrial scale. High CO₂ concentration was also tested, in order to maximize photosynthetic activity and photoautotrophic biomass production. In mixotrophic conditions, however, excess carbon dioxide stimulated photosynthesis but also blocked the metabolism of the organic substrate, likely because of inhibition of respiration. Finally microalgae cultivated under light-dark cycle in the presence of glycerol showed increased performances thanks to their ability to use organic substrates to support algal growth during the night, but only if CO₂ supply was stopped in the dark.

4.1. Introduction

Oil production from microalgae is one promising alternative to complement and eventually replace fossil fuels in next decades. Microalgae, in fact, are photosynthetic unicellular organisms which can grow at a much faster rate than plants and reach higher productivities (Chisti, 2008). Microalgal cultures have a number of additional advantages when compared with other bio-based renewable sources. First of all, unlike first-generation biofuels, extensive

microalgal cultivations do not compete with food crops for arable land, since they can be grown on marginal areas or in aquatic systems (Chisti, 2008). In addition, microalgae can reduce carbon dioxide emissions, by adsorbing CO₂ from combustion gases. In fact, atmospheric CO₂ concentration is limiting for algal growth and flue gas can be used as a cheap source with the double goal of supporting algal growth and reducing carbon dioxide released into the atmosphere, although SO₂ and NO_x might cause microalgae growth inhibition (Lee et al., 2002).

When algae are grown with CO₂ as the unique carbon source, light provides all the energy required for biomass production. Under autotrophic conditions, however, growth is often limited by light availability and, during the night, productivity is further reduced because of respiration losses. However, some phototrophic algae can also use organic carbon sources to support their growth and photoheterotrophy, or mixotrophy, is broadly defined as a growth regime in which CO₂ and organic carbon are simultaneously assimilated, with both respiratory and photosynthetic metabolism operating concurrently. From an industrial point of view, mixotrophy could support active growth within a photobioreactor, during night time, or in dark zones of the process, increasing the overall biomass productivity. If organic compounds supporting growth are derived from industrial or agricultural wastes, an increased productivity is also achieved at low cost. Additional potential environmental benefits are also present if wastewaters from municipal, agricultural and industrial activities are exploited as sources for organic molecules (Pittman et al., 2011).

In addition to the benefits in terms of biomass accumulation, the addition of organic carbon sources was also reported to stimulate lipids accumulation (Heredia-Arroyo et al., 2010). It is worth mentioning, however, that all studies analysing mixotrophic biomass and lipid production were performed in laboratory conditions and the possibility that the reported biomass yield can be maintained over long cultivation periods remains undemonstrated (Pittman et al., 2011).

Especially at the industrial scale, mixotrophy is likely to increase bacterial and fungal contaminations, a problem that can be controlled only in closed and strongly controlled systems such as photobioreactors (Lee and Zhang, 1999). The use of chlorination, antibiotics and herbicides could potentially reduce contamination, but in many cases they also inhibit microalgal growth.

In heterotrophic and mixotrophic cultures of microalgae the selection of carbon sources to be employed is essential, since they serve both as energy and carbon source for cell growth. Glucose is most commonly used for sustaining microalgae growing in the dark (Perez-Garcia et al., 2010) and was employed as carbon source in heterotrophic or mixotrophic culture of several microalgal species (Santos et al., 2010) reaching high production of both biomass and lipids (Xiong et al., 2010; Wan et al., 2011). From an industrial perspective, however, glucose is

not a suitable choice because of its high cost. Alternative carbon sources, derived as byproducts from other industrial processes, are preferable: these are the cases of acetate (Goksan and Gokpinar, 2010), ethanol (Lee, 2004) and glycerol (Herredia-Arroyo et al., 2010). Heterotrophic and mixotrophic growth using glycerol has been demonstrated for several algae, although the knowledge of glycerol metabolism is still limited, as reviewed by Perez-Garcia et al. (2010).

In this work, we tested the capability of *Chlorella protothecoides* and *Nannochloropsis salina* of using these organic substrates (ethanol, acetate and glycerol) as carbon source, by measuring and comparing growth kinetic parameters, maximum biomass concentration and lipid accumulation under autotrophic and mixotrophic conditions. In all experiments we also considered the effect of CO₂ supply, which is a major parameter for algal growth (Sforza et al., 2010). Interestingly, we found that with excess CO₂ concentration microalgae do not consume the organic substrate likely because of an inhibition of respiration. Thus, simultaneous CO₂ and organic compounds supply need to be finely optimized to achieve the best productivities in mixotrophy conditions.

4.2. Materials and Methods

4.2.1. Microalgae and media composition

Chlorella protothecoides 33.80 and *Nannochloropsis salina* 40.85 strains were obtained from SAG-Goettingen, Germany. *C. protothecoides*, a freshwater species, and was grown in BG11 medium (Rippka et al., 1979), with peptone 0.1% addition, following SAG indications. *N. salina*, a marine species, was cultured in sterilized sea salts 22 g/L solution enriched with f/2 Guillard solution as described in section 2.1.1., modified by adding an excess of nitrogen source (1.5 g/L of NaNO₃). For the experiments with different organic carbon sources, the basic media was modified as reported in table 4.1. The concentrations of organic substrates was selected on the basis of data reported in literature for each species (Liang et al., 2009; Xu et al., 2004; Li et al., 2007; Wood et al., 1999).

Table 4.1: Concentrations of organic substrates added to basic culture media.

Species	Substrate	% (w/v) in basic medium
<i>C. protothecoides</i>	Ethanol	0.4%
	Glycerol	1%
	Sodium acetate	0.8 %
<i>N. salina</i>	Ethanol	0.5%
	Glycerol	1%
	Sodium acetate	0.5 %

4.2.2. Growth analysis

Growth experiments were performed both in Erlenmeyer flasks (batch system) and in 0.25-L glass bottles under a continuous enriched CO₂ feed flow (semibatch system), as described in section 2.3. Algal growth kinetics were measured by daily changes in optical density and cells number as described in section 2.1.

4.2.3. Analytical methods

The lipid content was determined by staining the algal cell suspension with Nile Red (NR) dye as described in section 2.1.

Glycerol consumption in the medium was determined as residual concentration in filtered medium by using a spectrophotometrical analytical kit provided by Steroglass Srl, Italy, based on phosphorylation of glycerol by glycerol kinase and subsequent oxidation by glycerol-3-phosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. The reaction of hydrogen peroxide with 4-aminofenazole and TOOS, catalyzed by peroxidase, generated a purple color (Beutler, 1984).

4.3. Results and discussion

4.3.1. Effect of organic substrates on growth

This study on microalgae mixotrophy was performed on two different species: *C. protothecoides* and *N. salina*. The former has already been shown to have a nutritional flexibility (auto, hetero, and mixotrophic) together with a high biomass productivity and the ability of accumulating high amounts of lipids (Santos et al., 2010). *N. salina*, on the contrary, is known to have a good biomass and lipid productivity in photo-autotrophic conditions (Rodolfi et al., 2009; Sforza et al., 2010) but mixotrophic growth of *N. salina* was reported only with glucose (Wood et al., 1999; Das et al., 2011) and its phenomenology is still far from being clearly understood.

C. protothecoides was initially grown in Erlenmeyer flasks under different mixotrophic conditions using ethanol, acetate and glycerol as organic substrates, to test its ability to import and metabolize these molecules. In figure 4.1, growth curves are reported and compared to a control curve with no added substrates. It is noteworthy that this control cannot be considered completely autotrophic, since peptone is present in the medium, as recommended by the culture collection (SAG-Goettingen, Germany). In the absence of peptone, in fact, cells did not grow significantly.

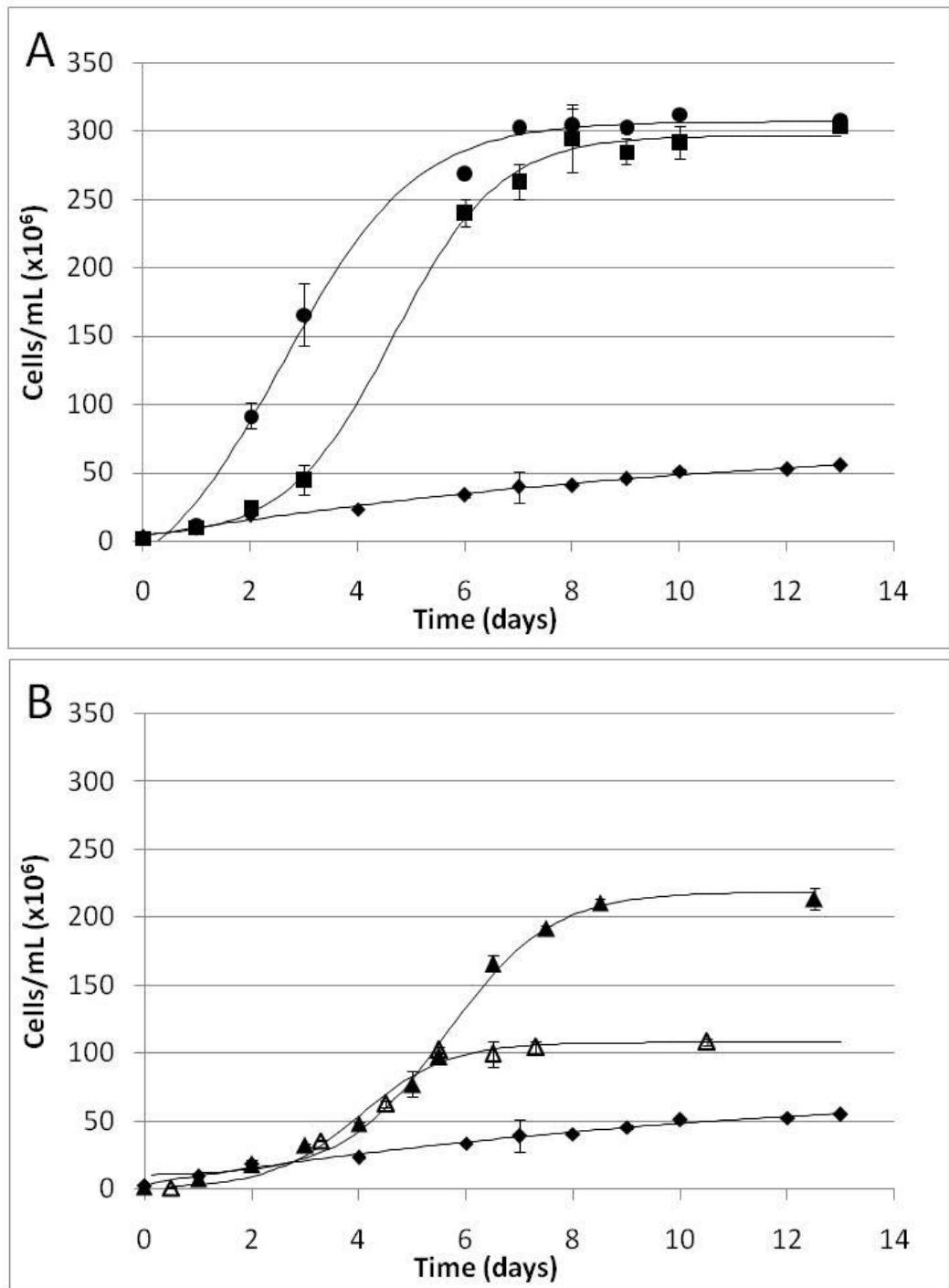


Figure 4.1. Growth curves of *C. protothecoides* under mixotrophic conditions with different sources of carbon: (■) ethanol; (●) sodium acetate; (♦) control (4.1A). In fig.4.1B growth curves of *C. protothecoides* with glycerol (1% W/V) as substrate, under mixotrophic (▲) and heterotrophic (△) conditions, compared to control (♦) are reported. Peptone (0,1% W/V) was added to the culture medium in all experiments. These growth experiments were done in Erlenmeyer flasks.

Table 4.2. Specific growth rate (μ) of *C. protothecoides* and *N. salina* in batch flasks experiments with different sources of carbon: ethanol (0,4% W/V) ; sodium acetate (0,8% DW) compared with control. Maximum biomass concentration data refers to the amount obtained at the end of the stationary phase of growth.

Substrate	<i>C. protothecoides</i>		<i>N. salina</i>	
	μ (d ⁻¹)	Max. biomass conc. (g/L)	μ (d ⁻¹)	Max. biomass conc. (g/L)
Ethanol	0.93±0.02	3.25±0.1	0.39±0.2	0.430±0.2
Sodium acetate	1.45±0.1	3.29±0.2	0.02	0.05
Control	0.30±0.09	0.52±0.1	0.32±0.2	0.78±0.01

Table 4.3. Specific growth rate (μ) of *C. protothecoides* and *N. salina* in flasks experiments. Glycerol consumption and maximum biomass concentration data refer to the amount obtained at the end of the stationary phase of growth. Mixotrophic and heterotrophic experiments were done by adding glycerol 1% W/V as organic substrate.

Growth conditions	<i>C. protothecoides</i>		
	μ (d ⁻¹)	Max. biomass conc. (g/L)	Glycerol consumption (%)
Mixotrophy (Glycerol 1%)	0.91±0.1	2.67±0.3	67.1
Heterotrophy (Glycerol 1%)	0.87±0.01	1.10±0.2	40.4
<i>N. salina</i>			
	μ (d ⁻¹)	Max. biomass conc. (g/L)	Glycerol consumption (%)
Mixotrophy (Glycerol 1%)	0.36±0.2	0.430±0.2	85.3
Heterotrophy (Glycerol 1%)	0.01	0.05	0

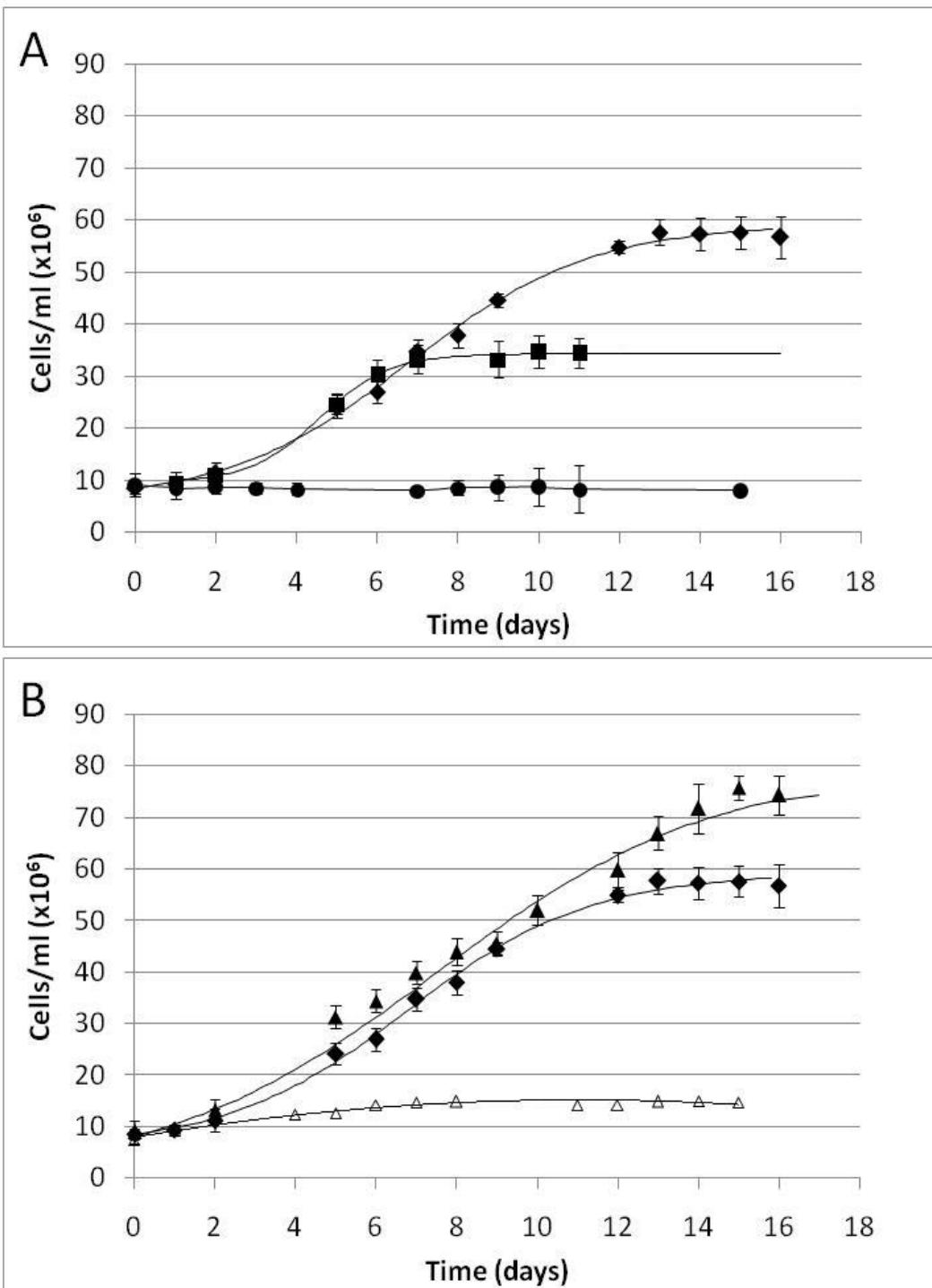


Figure 4.2. Growth curves of *N. salina* under mixotrophic conditions with different sources of carbon: (■) ethanol; (●) sodium acetate; (♦) control. In fig. 4.2B growth curves of *N. salina* with glycerol (1% W/V) as substrate, under mixotrophic (▲) and heterotrophic (△) conditions, compared to control (♦) are reported. These growth experiments were done in Erlenmeyer flasks.

As reported in figure 4.1A and table 4.2, *C. protothecoides* cultures in the presence of both ethanol and sodium acetate showed an enhanced growth. In particular, the specific growth rates were 0.93 d^{-1} with ethanol and 1.45 d^{-1} with acetate as compared to 0.30 d^{-1} for the control. Also final biomass concentration was higher, especially in the case of acetate (3.29 g/L vs. 0.52 g/L of control). Thus, mixotrophic growth of *C. protothecoides* can increase the biomass productivity by about six folds, and triplicate its growth rate by using the widely available substrates tested here. From an industrial perspective, glycerol would be a highly suitable carbon source since it is a byproduct of transesterification reaction in the biodiesel production process. Thus, an external recycle of glycerol could contribute to the economical feasibility of algal biomass production and would avoid devaluation on the market of this organic compound. For this reason glycerol was also tested for mixotrophic cultures of *C. protothecoides*, yielding again interesting results: when the medium was supplemented with 1% glycerol, in fact, growth was three folds faster than the control, as reported in figure 4.1B and table 4.3. Glycerol was indeed metabolized by the alga as shown by the fact that up to 60% of the glycerol initially present in the medium was consumed (table 3). While the measured growth rate was similar to that obtained with other substrates, a lag-phase of about two days was observed, suggesting that cells needed some metabolic adaptation to be able to metabolize glycerol. *C. protothecoides* was also cultivated in the dark under fully heterotrophic conditions with glycerol as carbon source (figure 4.1B and table 4.3). The results showed a growth rate in the dark similar to autotrophic control ($\mu= 0.87\text{ d}^{-1}$), but a lower biomass yield, with a final concentration of only 1.1 g/L. This finding is consistent with a smaller substrate consumption (about 40%, table 4.3) in the absence of light, as reported also for other species such as *Chlorella vulgaris* (Martinez and Orus, 1991). Thus, mixotrophic growth was stronger than both autotrophic and heterotrophic.

Analogous experiments were also performed using *N. salina* (figure 4.2A and table 4.2). In this case, ethanol supplied cultures showed a small increase in specific growth rate with respect to the autotrophic control (0.39 d^{-1} and 0.32 d^{-1} respectively), but with a lower final cell concentration. Instead, *N. salina* growth was strongly inhibited by acetate, which was turned out to be toxic for the cells in the tested concentration. On the contrary, glycerol (figure 4.2B) was effective in supporting *N. salina* growth, which showed a rate similar to the autotrophic control but with a higher final concentration (table 4.3). Differently from *C. protothecoides*, *N. salina* was found unable to grow in the dark under fully heterotrophic conditions.

4.3.2. Mixotrophic growth in the presence of excess CO₂

The results presented above showed that both species investigated are able to grow mixotrophically with glycerol. In previous experiments CO₂ was present at the atmospheric concentration, while it is well known that carbon dioxide in excess strongly enhances biomass productivity of algae autotrophic photobioreactors. The improvement of photosynthetic efficiency should lead to a further improvement of growth rate and for this reason we cultivated the two algal species with air enriched with excess CO₂ (5% v/v), as previously tested by Sforza et al. (2010). As expected for both species, excess CO₂ stimulated autotrophic growth, with higher specific growth rates (Figure 4.3, Table 4.4 and 4.5).

C. protothecoides in CO₂ enriched experiments, however, showed very similar growth rates both in the presence (mixotrophic) and in the absence (autotrophic) of glycerol (1.72 d^{-1} and 1.67 d^{-1} , respectively). Also the total biomass productivity was not affected by the presence of organic substrate and glycerol addition even caused a small reduction in the final concentration. Consistently with this negligible effect, only a small fraction of the glycerol present in the medium (4%) was consumed, as reported in table 4.4. These results may be explained by the presence of peptone in the medium which might be preferentially metabolized over glycerol. To verify this point *C. protothecoides* was grown without peptone; with excess CO₂ we observed significant growth (figure 4.3A), although rates were significantly smaller than those in the peptone added medium (0.92 d^{-1} against 1.26 d^{-1} , respectively), probably because peptone is also a source of nitrogen, another key nutrient for algal growth. Glycerol addition did not affect results also in the absence of peptone, confirming that its presence is useless.

These data thus show that CO₂ in excess stimulates microalgal photoautotrophic growth, maximizing photosynthetic reactions, but it also reduces the efficiency of organic substrate uptake, which remains in the culture medium. Similar results were obtained with *N. salina*, where CO₂ in excess improved autotrophic growth but the glycerol present in the medium was not metabolized and even slightly inhibited the final biomass concentration likely because of osmotic effects (Figure 4.3B, table 4.5).

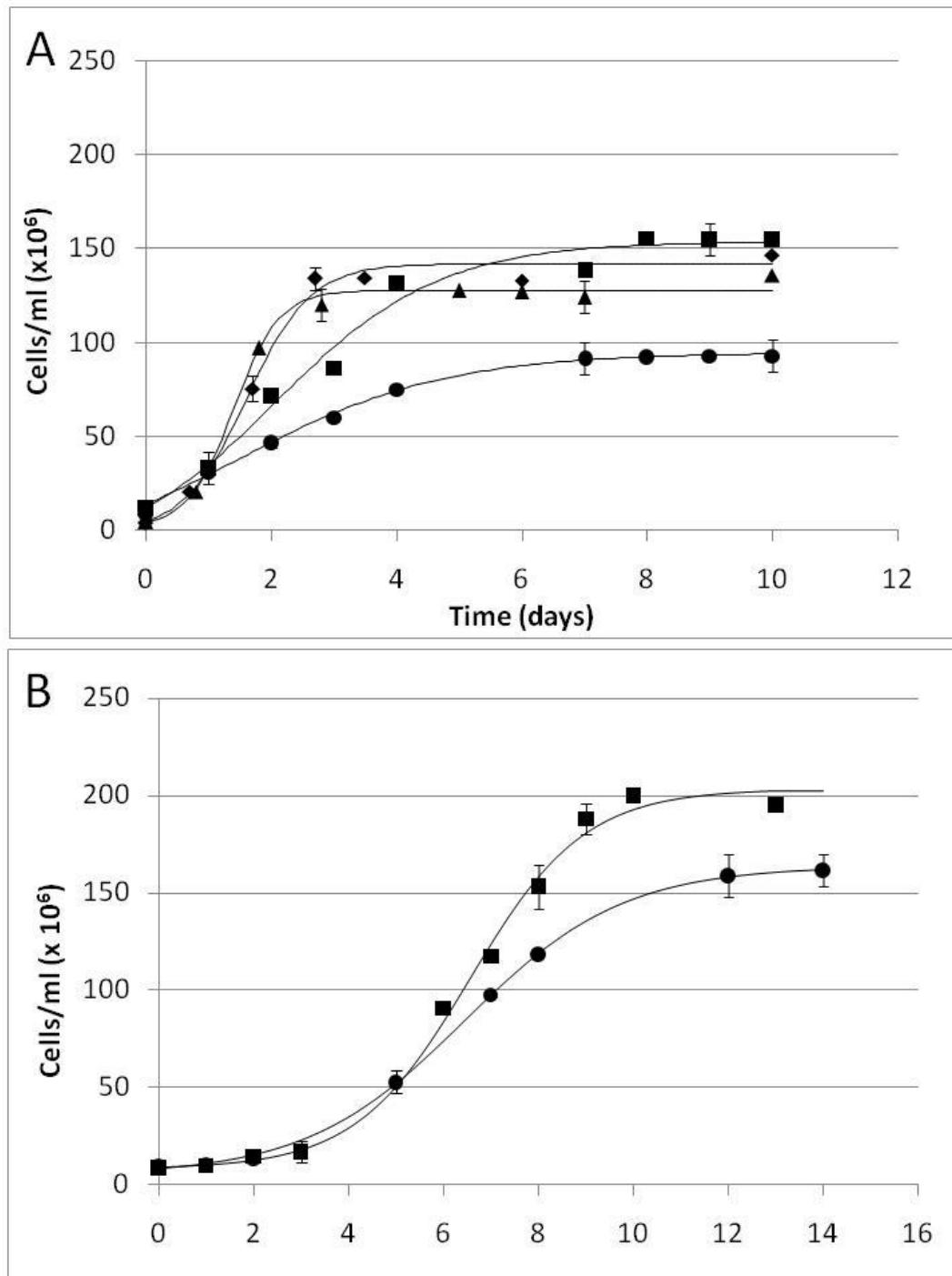


Figure 4.3. Effect of CO_2 (5% v/v in bubbling air) on growth curves of *C. protothecoides* (fig A) and *N. salina* (fig B) under different culture conditions: autotrophic (■) and glycerol 1% W/V (●). For *C. protothecoides* growth curves with peptone 0,1% W/V(▲), peptone 0,1% W/V- glycerol 1% W/V (◆) are also reported. All experiments were done in bubbling system.

Table 4.4. Specific growth rate (μ) of *C. protothecoides* cultured in bubbling system. Glycerol consumption and maximum biomass concentration data refer to the amount obtained at the end of the stationary phase of growth. Mixotrophic and heterotrophic experiments were done by adding glycerol 1% W/V as organic substrate.

Culture conditions	<i>C. protothecoides</i>		
	μ (d ⁻¹)	Glycerol consumption (%)	Max. biomass conc. (g/L)
Autotrophic	1.26±0.10	--	3.60 ±0.10
Peptone	1.67±0.08	--	2.78±0.10
Glycerol	0.92±0.09	17.6	2.08±0.03
Glycerol + Peptone	1.72±0.20	4.2	3.22±0.20

Table 4.5. Specific growth rate (μ) of *N. salina* cultured in bubbling system. Glycerol consumption and maximum biomass concentration data refer to the amount obtained at the end of the stationary phase of growth. Mixotrophic and heterotrophic experiments were done by adding glycerol 1% W/V as organic substrate.

Culture conditions	<i>N. salina</i>		
	μ (d ⁻¹)	Glycerol consumption (%)	Max. biomass conc. (g/L)
Autotrophic	0.55±0.05	--	2.53 ±0.01
Mixotrophic	0.36±0.1	3.2	2.10±0.2

4.3.3. Exploitation of mixotrophy to support growth with a night/day light cycle

Results presented above showed that *N. salina* and *C. protothecoides* are able to metabolize glycerol, but this ability is inhibited by a CO₂ excess. It should also be considered that previous experiments were performed under continuous illumination while in outdoor photobioreactors microalgae use light only during daytime. One possibility to maximize growth is to sustain algal growth overnight by providing an organic carbon source which, even if not metabolized during the day, could support growth in the night. To verify the feasibility of this possibility, we cultivated algae under night-day cycle of 12:12h, both under autotrophic and mixotrophic conditions, always supplying CO₂ in excess (figure 4.4). As expected, autotrophic specific growth rates were slower for both species with respect to those with continuous light (0.68 d⁻¹ for *C. protothecoides* and 0.367 d⁻¹ for *N. salina*, instead of 1.26 d⁻¹ and 0.55 d⁻¹ respectively, tables 4.6 and 4.7). However, the presence of a glycerol enriched medium did not increase any of these values, and growth was even inhibited. In fact, both *C. protothecoides* (figure 4.4A) and *N. salina* (figure 4.4B), reached a lower final concentration of biomass in the presence of glycerol with respect to the autotrophic culture in a 12:12 h night-day cycle. Consistently, the organic substrate was not consumed, similarly to what was previously observed with continuous illumination.

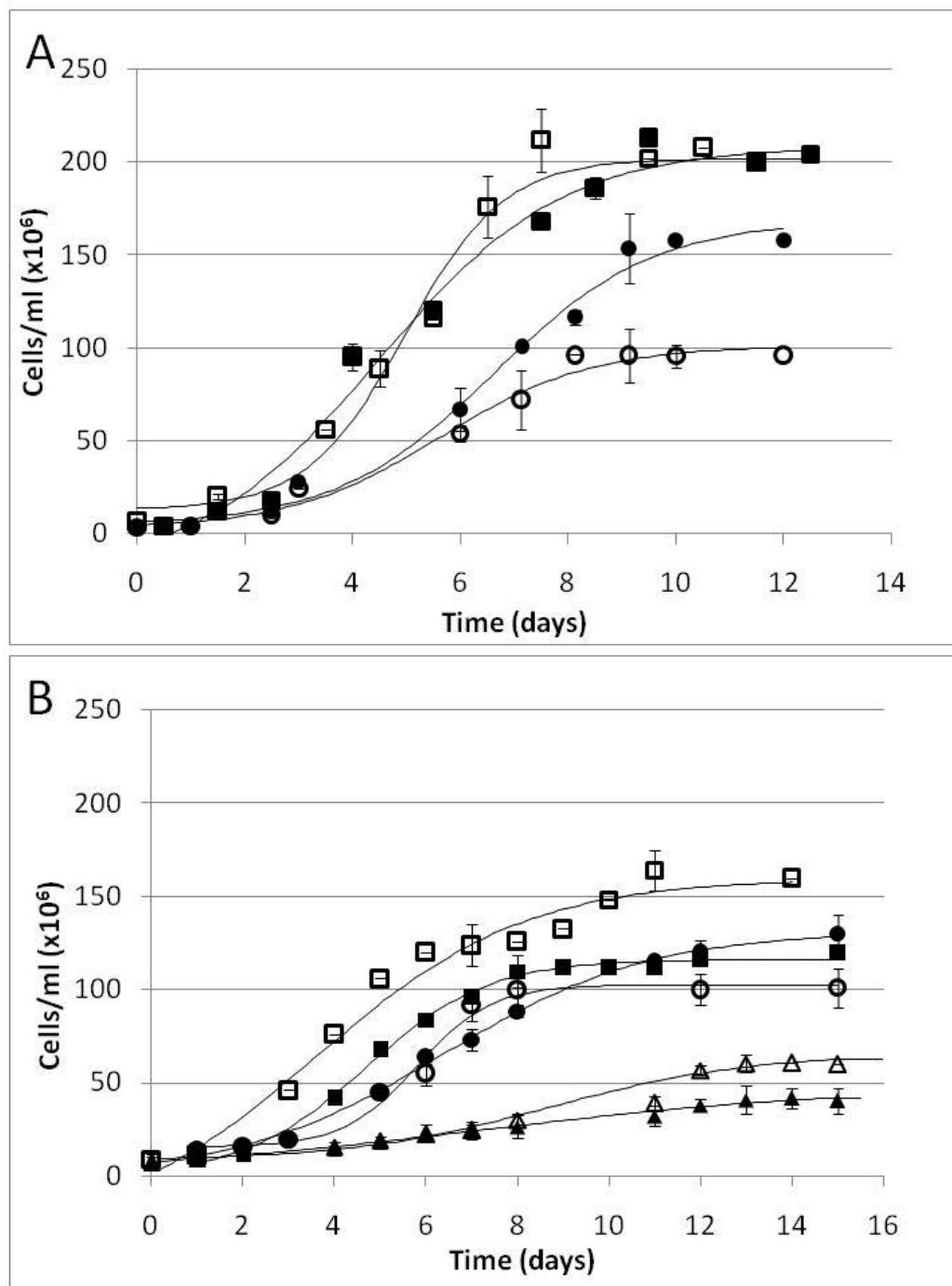


Figure 4.4. Growth curves of *C. protothecoides* (fig A) and *N. salina* (fig B) under night-day cycle (12h-12h) in bubbling system with added 5% v/v CO₂ in air under autotrophic (●), and mixotrophic (○) conditions. In autotrophic (■), and mixotrophic (□) curves, CO₂ (5% v/v) was supplied only during light period. During night, cultures are bubbled with CO₂ deprived air. All experiments reported were done in bubbling system and mixotrophic experiments were done by adding glycerol 1% W/V to the culture medium. For *N. salina* experiments in autotrophy (▲), and mixotrophy (Δ) under atmospheric conditions are also reported.

These observations cannot be explained only by the CO₂ stimulation of photosynthetic reactions since, photosynthesis is not active in the dark. On the contrary they suggest the presence of a CO₂ inhibitory effect on glycerol metabolism. This is true for both species considered and, since they are not evolutionarily related, this is likely to be a widespread property of algae.

To further investigate this point, we reduced the CO₂ concentration in bubbling air during the night down to the atmospheric concentration of 0.03%, to verify if the ability of metabolize glycerol could be restored. For both species, even with low CO₂ concentration there was no substantial glycerol uptake (tables 4.6 and 4.7).

Table 4.6. Specific growth rate (μ) of *C. protothecoides* cultured in bubbling system under night-day cycle and different cycle of CO₂ supplying.. Glycerol consumption and maximum biomass concentration data refer to the amount obtained at the end of the stationary phase of growth. Mixotrophic experiments were done by adding glycerol 1% W/V as organic substrate.

CO ₂ flow conditions (% v/v in air)	Culture conditions	μ (d ⁻¹)	Glycerol consumption (%)	Max. biomass conc. (g/L)
CO ₂ 5% 24h	Autotrophic	0.680±0.04	--	3.12 ±0.05
	Mixotrophic	0.640±0.05	0	2.01±0.1
CO ₂ 5% in light – 0.03% in dark	Autotrophic	0.664±0.06	--	2.99 ±0.01
	Mixotrophic	0.627±0.08	2	2.13±0.1
CO ₂ 5% in light – 0% in dark	Autotrophic	0.868±0.09	--	2.78±0.1
	Mixotrophic	0.867±0.1	8	2.67±0.2

Such a result is an apparent contrast with what reported in figures 4.1 and 4.2. However, it should be considered that while experiments in figures 4.1 and 4.2 refer were carried out in flasks, in the latter case air was bubbled through the culture. Therefore, although CO₂ concentration is the same, its mass transfer in the medium is far higher in this latter case.

To verify this hypothesis experiments were repeated using CO₂-free air during the night and in this case both species were able to shift their metabolism to heterotrophy during the night and metabolize glycerol. In this case, *C. protothecoides* and *N. salina* increased their growth rate to 0.867 d⁻¹ and 0.61 d⁻¹ respectively, and significant glycerol consumption was also measured (about 8% an 20% respectively, tables 4.6 and 4.7).

Table 4.7. Specific growth rate (μ) of *N.salina* cultured in bubbling system under night-day cycle and different cycle of CO_2 supply. Glycerol consumption and maximum biomass concentration data refer to the amount obtained at the end of the stationary phase of growth. Mixotrophic experiments were done by adding glycerol 1% W/V as organic substrate.

CO_2 flow conditions (% v/v in air)	Culture conditions	$\mu (\text{d}^{-1})$	Glycerol consumption (%)	Max. biomass conc. (g/L)
CO_2 5% 24h	Autotrophic	0.367±0.01	--	1.40 ±0.1
	Mixotrophic	0.354±0.01	0	1.01±0.14
CO_2 5% in light period - 0.03% in dark	Autotrophic	0.399±0.01	--	0.99 ±0.01
	Mixotrophic	0.335±0.01	2	0.7±0.12
CO_2 5% in light - 0% v/v in dark	Autotrophic	0.53±0.01	--	1.30±0.2
	Mixotrophic	0.613±0.01	19.8	2.05±0.2

These results show that in the presence of a day/night cycle, glycerol addition stimulates growth but only if CO_2 is not in excess during the dark phases.

The presence of CO_2 during the dark period seems to influence the growth rate also in full autotrophy. In fact, under 12:12 h night-day cycle cultures bubbled with CO_2 -free air during the night (figure 4.4) showed better growth rates than the ones continuously provided with 5% CO_2 . This result suggests that CO_2 inhibitory effect is likely not restricted to glycerol but rather a more general inhibitory effect on respiration. Consistently, respiration inhibition by excess of CO_2 is known for plants (Amthor et al., 1992) but it has never been reported before for microalgae. The observation that an inhibition of respiration might reduce biomass accumulation might seem counterintuitive since respiration should consume organic carbon molecules fixed during the day. However, it should be considered that respiratory and photosynthetic metabolism are strongly connected and it has been shown that inhibition of respiration also reduces photosynthetic efficiency, especially in the transition from the dark to illumination (Noctor et al., 2004). An alternative explanation might be that excess CO_2 during the night is not metabolized and it might alter pH of the medium and reduce growth.

4.3.4. Effect of mixotrophy on lipid accumulation

In the perspective of developing a large scale biodiesel production from microalgae, the effect of mixotrophy was investigated not only on biomass productivity but also on lipids accumulation. It has already been shown that heterotrophy or mixotrophy could significantly increase the amount of lipid in the cells of *C. protothecoides* when glucose was used as substrate (Xiong et al., 2010). In our experiments we confirm that *C. protothecoides* accumulates more lipids under mixotrophic conditions (21% under mixotrophy instead of 12% in autotrophy, as reported in table 4.8), although the lipid content remained far lower than values obtained by other authors (about 55%, as reported by Xu et al., 2006), where only glucose was used as substrate. Also, we found that mixotrophy was less effective than

nitrogen limitation to induce lipids accumulation which in the latter case reached 35% DW (table 4.8).

Table 4.8. Lipid content of *C. protothecoides* under different growth conditions. Maximum lipid concentration refers to results obtained at the end of stationary phase.

	Max. lipid conc. (%DW)
Mixotrophic (glycerol 1%)	21.3±2.5
Autotrophic	11.5±3.1
Autotrophic N-deprived	35.8±1.5

Lipid accumulation is also well known to be induced by nitrogen limitation also for *N. salina* (Rodolfi et al., 2009; Sforza et al., 2010). Our results showed that mixotrophy could induce lipid accumulation in *N. salina* even when nitrogen is in excess (1.5 g/L of NaNO₃): about 15 days after the *inoculum*, the lipid content increased up to about 50% of DW, , as reported in figure 4.5.

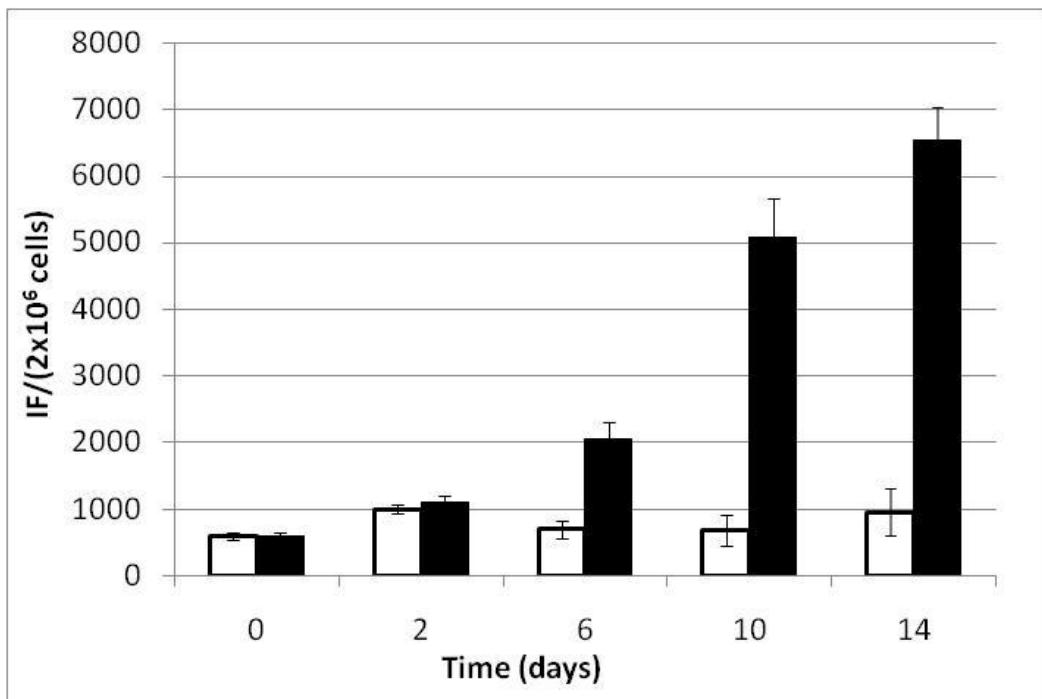


Figure 4.5. Fluorescence intensity values of Nile-Red stained cells of *N. salina* during growth curves under autotrophic (□) and mixotrophic (■) conditions (glycerol 1% W/V). Data refers to growth curves under atmospheric CO₂ concentration.

From an industrial perspective, this could simplify the process of oil production from microalgae, because only one step would be required to produce simultaneously biomass and lipids, instead of a two step process proposed in the case of autotrophic systems (Rodolfi et al., 2009; Sforza et al., 2010). It should be pointed out, however, that glycerol is able to affect lipid accumulation only when is actively consumed, i.e. only when CO₂ concentration is low.

4.4. Final remarks

In this chapter, it has been verified that *C. protothecoides* is able to grow in mixotrophic cultures, and may use organic substrates alternative to sugar, such as ethanol, acetate and glycerol. *N. salina* may also use ethanol or glycerol while its growth is strongly inhibited by acetate. We focused in particular on glycerol since this is a byproduct of biodiesel production and could be recycled in an industrial algal biomass production process.

Microalgae cultures need to be supplied with high CO₂ concentrations to maximize their photosynthetic productivity, but we showed that these conditions also inhibit mixotrophy, likely because of an inhibition of respiration. Thus, the ideal compromise for an outdoor photobioreactor working with a night-day cycle to reach the maximal biomass productivity will be to have an autotrophic growth with excess CO₂ during the day and heterotrophic growth in glycerol without adding CO₂ during the night.

We also observed that the addition of glycerol during the day is able to increase the lipid content in both algal species. Although higher lipids contents have been obtained with nitrogen deprivation, the addition of an organic substrate is preferable for the design of an industrial process. Thus the use of mixotrophy, provided that CO₂ supply is turned off during the night, represents a good compromise between fast growth and high lipid content in *N. salina* and *C. protothecoides* cultures.

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CHAPTER 5

EXPLOITATION OF INDUSTRIAL BYPRODUCTS AS NUTRIENTS FOR MICROALGAL GROWTH

In this chapter, experiments carried out in order to verify the possibility to exploit industrial byproducts to feed microalgal cultures are reported.

From the perspective of using industrial byproducts from actual industrial facilities, the sources available on site are to be considered. Consequently, in some cases, freshwater species are needed when salt water are not locally available. To this aim, we selected a freshwater species able to grow fast and accumulate lipids, and we verified the capability of that species to exploit industrial byproducts.

5.1. Introduction

As reported in chapter 3, worldwide several researchers claim to possess technologies for the commercial production of biodiesel from microalgae, and many new companies have been recently started (Singh and Gu, 2010; Zijffers et al., 2010; Grobbelaar 2010), but no industrial plants finalized to produce oil from microalgae are operated in the world yet (Singh and Gu, 2010) and process feasibility has not been demonstrated for large scale production as well. To this aim there is the need to develop industrial pilots and do on-field tests in order to better understand this process, and to give a contribution towards its industrialization and large-scale application. One of the previously cited advantages of using microalgae is related to their ability to use higher amount of CO₂ taken from air or, alternately, the possibility to consume flue gases to feed a microalgal culture.

In a theoretical scenario, gas from the fossil fuel combustion system and nutrients are added to a photobioreactor where microalgae utilize sunlight to photosynthetically convert the CO₂ into compounds of high commercial values, biofuels or mineralized carbon, thus achieving sequestration of CO₂. Using a stream from a combustion plant in a microalgal-based system has several advantages. High purity CO₂ gas is not required for algal culture and flue gas

containing varying amounts of CO₂ can be fed directly into it. This can also simplify the CO₂ separation from flue gas significantly. Flue gas can be used as a cheap source with the double goal of simultaneously supporting algal growth and reducing carbon dioxide released into the atmosphere, although there are some evidences that SO₂ and NO_x might cause microalgal growth inhibition (Lee et al., 2002).

The objective of this project was to select a species from freshwater, access the effect of several operating variables on microalgae growth and lipids accumulation, and test the ability of these species to use byproducts of an actual production process.

5.2. Materials and methods

5.2.1. Microalgae, media composition and methods

The microalgal species were selected on literature data basis (Krienitz et al. 2006, Dayananda et al. 2007, Gouveia et al. 2009). In particular we screened *Nannochloropsis limnetica*, *Botryococcus braunii*, *Neochloris oleabundans*, *Chlorella protothecoides*, Strain7 and Strain20 (specific names of these species are not reported for industrial secrecy reasons; they are two strains of the same species). All species are fresh water and were cultivated in BG11 medium as described in section 2.1. The suppliers of strains are reported in table 5.1.

Table 5.1 Microalgal strain used and their source.

Species	Strains	Source
<i>Botryococcus braunii</i>	33.80	SAG - Culture Collection of Algae at Goettingen
<i>Botryococcus braunii</i>	807.1	SAG - Culture Collection of Algae at Goettingen
<i>Nannochloropsis limnetica</i>	18.99	SAG - Culture Collection of Algae at Goettingen
<i>Neochloris oleabundans</i>	1185	UTEX- The Culture collection of Algae
Strain7		SAG - Culture Collection of Algae at Goettingen
Strain20		SAG - Culture Collection of Algae at Goettingen

For growth analysis and analytical methods, refer to section 2.1.

5.3. Results and discussion

5.3.1. Screening of the species

All growth curves were measured in the bubbling system described in section 2.2.1, in order to supply a non limiting CO₂ concentration (5% v/v CO₂ in air). The results, reported in table 5.2 and in figures 5.1, 5.2 and 5.3, were quite different for the species considered.

In particular, we found that *N. limnetica* is not able to grow in the presence of an excess of CO₂. This is probably due to the high susceptibility to pH changes in the media bubbled with CO₂-enriched air, despite of the capability of this species of growing under atmospheric conditions (see section 2.3). For this reason, this species was not considered for further studies, due to its weakness to environmental changes.

Among the other species, *Strain20* (figure 5.2) showed the highest growth rate, with a kinetic constant of 0.93 d⁻¹ and a remarkable final concentration (1.85 g/L). Anyway, the two strains named *Strain7* and *20* were strictly similar, with respect to both growth rate and biomass concentration. The two strains of *B. braunii* showed a slower growth rate, (0.42 d⁻¹ for strain 807-1 and 0.37 d⁻¹ for strain 33.80), even though they reached a final concentration about three times higher than under atmospheric conditions (see chapter 2). Interesting results were also obtained for *N. oleoabundans*, with a specific growth rate of 0.64 d⁻¹ and a final concentration of 0.22 g/L.

5.3.2. *Lipid content of screened species*

Concerning the lipid content of the species screened, data of fluorescence intensity are reported in figure 5.1 for *B. braunii* 807-1 and *N. oleoabundans*. On the opposite, for *Strain7* and *Strain20* the Nile Red staining was not used, because we found that there was no correlation between IF data and lipid content for that species, maybe due to a poor permeability of the cell wall of this species to the dye.

B. braunii 807-1 showed a considerable lipid content of about 35 % (calculated from correlation with IF data, 11000). Similarly, strain 33.80, that displayed a constant concentration of lipid during different phases of growth, reached a lower lipid content, of about 30% DW. However, this value is lower than data reported in literature (61% DW, Metzger and Largeau, 2005). One possible explanation of these results is that *B. braunii* seems to have the capability of secreting lipid droplets, and this could justify our data, since the soxhlet technique can detect only lipid contained in the biomass.

Concerning *N. oleoabundans*, an IF of 9000 corresponds to a lipid content of 24%. Anyway, this species showed a singular behavior of the IF trend. In fact, as reported in figure 5.1, in the early stationary phase, *N. oleoabundans* started to accumulate lipids, reaching the maximum content on the 8th day. After this point, the lipid content drastically decreased. This behavior was already observed for this species by Gouveia and Oliveira (2009), but the biological reason is not clearly understood. Anyhow, the trend of lipid accumulation and following consumption was found reproducible in all the experiments carried out.

For *Strain7* and *20*, soxhlet extraction of biomass at the end of growth curves showed that the strain 7 is able to accumulate a lipid content of 41% DW, while *Strain20* only reached a concentration of 25% DW. These results demonstrated that, even under similar conditions,

there are remarkable differences not only among different species, but also among different strains, making the screening of microalgae very important from an industrial perspective.

Table 5.2. Specific growth rate (μ) and duplication time of microalgae screened in bubbling system. Maximum concentration data refer to the biomass amount obtained in stationary growth phase.

Species	μ (days $^{-1}$)	t_d (days)	Max. biomass conc. (g/L)	Lipid content (% DW)
<i>B. braunii</i> 807-1	0.42	1.65	1.18	35
<i>B. braunii</i> 33.80	0.37	1.87	1.32	30
<i>N. oleoabundans</i> 1185	0.64	1.08	1.22	24
Strain7	0.81	0.86	1.91	41
Strain20	0.93	0.75	1.85	25

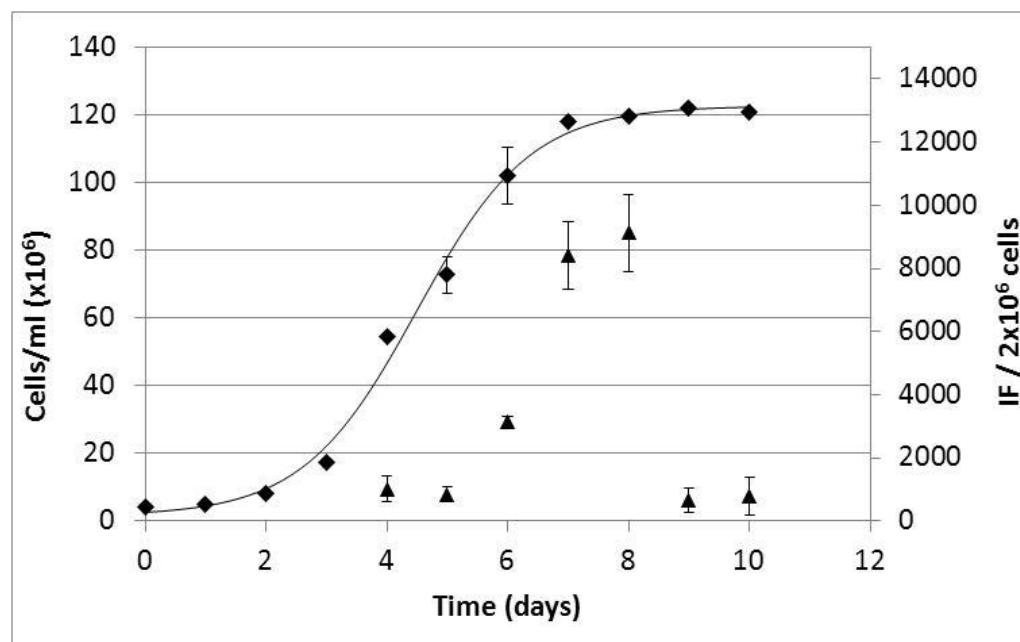


Figure 5.1 Growth curve (♦) and IF of Nile Red staining (▲) of *N. oleoabundans*, under autotrophic conditions and minimum media composition. These runs were performed in bubbling tubes.

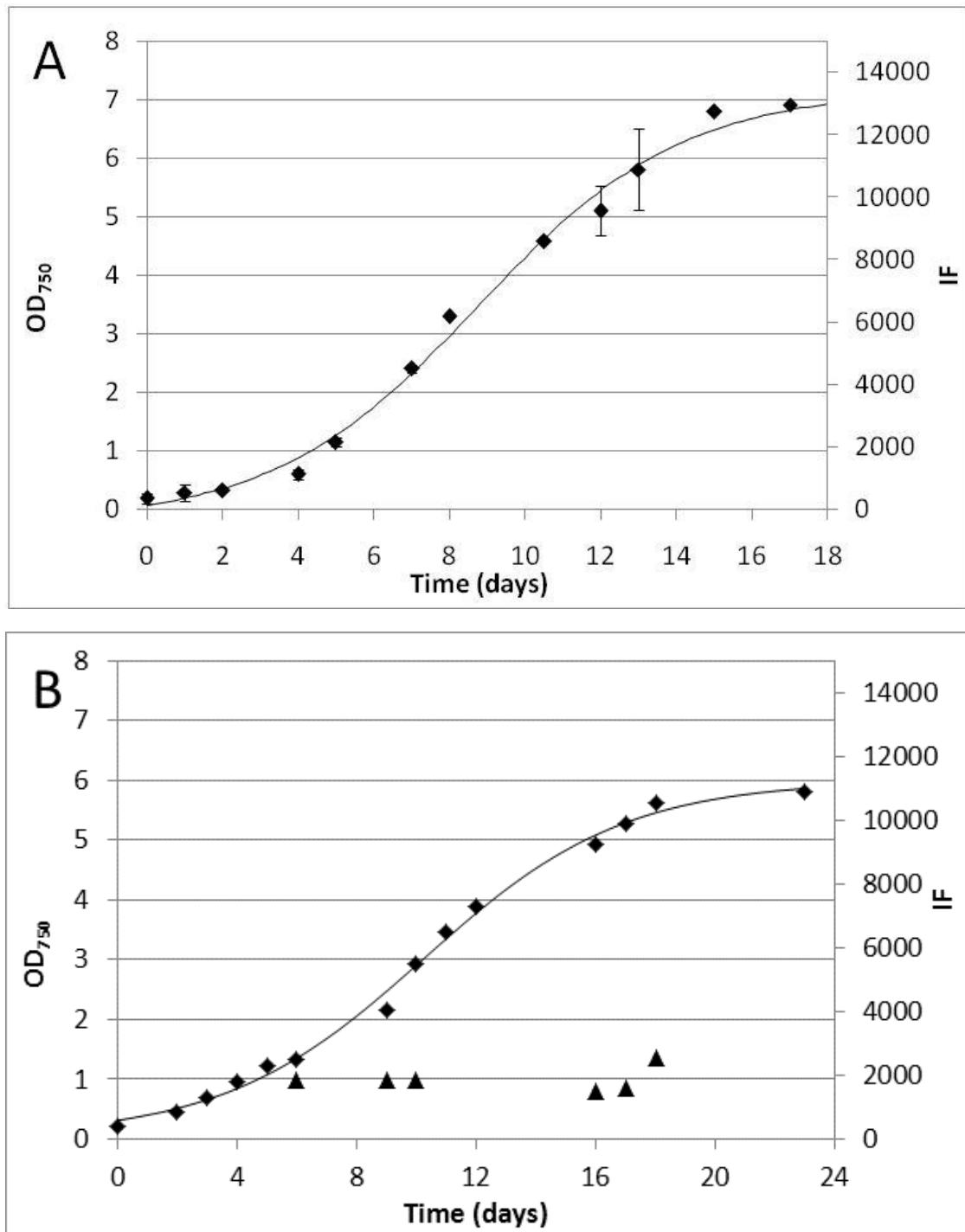


Figure 5.2 Growth curve (◆) and IF of Nile Red staining (▲) of *B. braunii* 807-1(A) and *B. braunii* 33.80 (B), under autotrophic conditions and minimum media composition. These runs were performed in bubbling tubes.

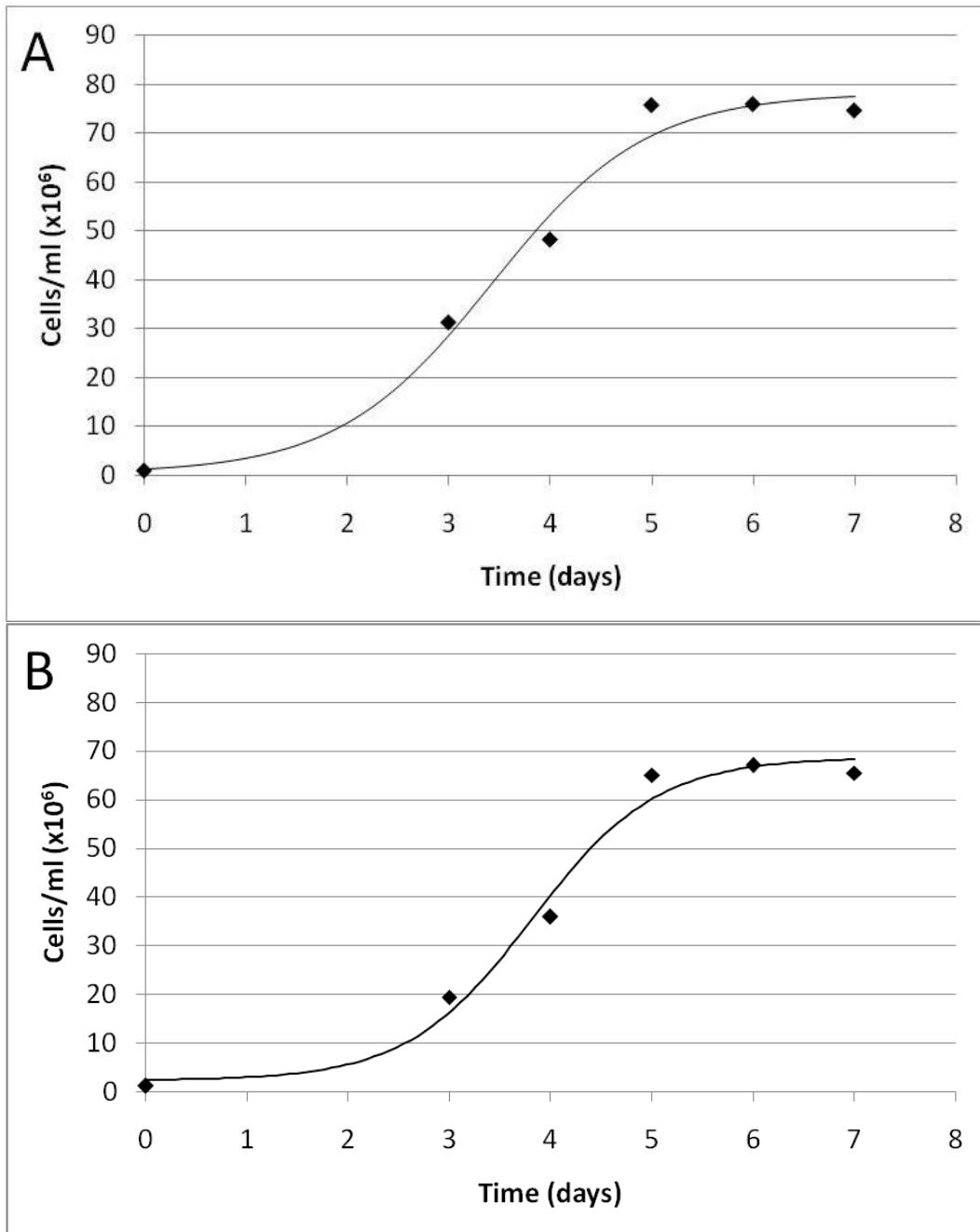


Figure 5.3 Growth curve (♦) of Strain20 (A) and Strain7 (B), under autotrophic conditions and minimum media composition. These runs were performed in bubbling tubes.

5.3.3. Optimization of lipid content by N-deprivation

In order to enhance the lipid content of species studied, nitrogen deprivation experiments were carried out. It's well known that N deprivation could stimulate a lipid accumulation in several species as we have already reported in chapter 3 for *N. salina*.

Concerning the two strains of *B. braunii*, only one of them (strain 33.80) showed an increased lipid content after N deprivation, reaching a remarkable concentration of about 57% (DW). On the opposite for strain 807-1, the reproducibility of experiments was lower, and an unexplained loss of vitality was found. This behavior, observed also in other conditions of

growth, might be explained as a seasonal control of biological cycles, but this is not clearly understood and demonstrated for microalgae. Anyway, under chemical stress conditions, for this strain an increased lipid content of 47% DW was measured.

The N deprivation in *N. oleabundans* confirmed the unusual trend of lipid accumulation and consumption, as already reported in section 5.3.2. In fact, as reported in figure 5.4, during the stationary phase, lipids were alternately synthesized and consumed, with a reproducible pattern (this experiment was repeated three times exactly at the same conditions).

On the sixth day of growth, the culture was centrifuged and resuspended in a N depleted medium. The IF data increased up to 12000 at the 11th day, reaching a lipid content of about 25% DW, that was consumed after the 12th day. Subsequently, a new phase of lipids accumulation started, and the maximum observed was higher than the previous peak, with a lipid accumulation of 35%DW on the 19th day.

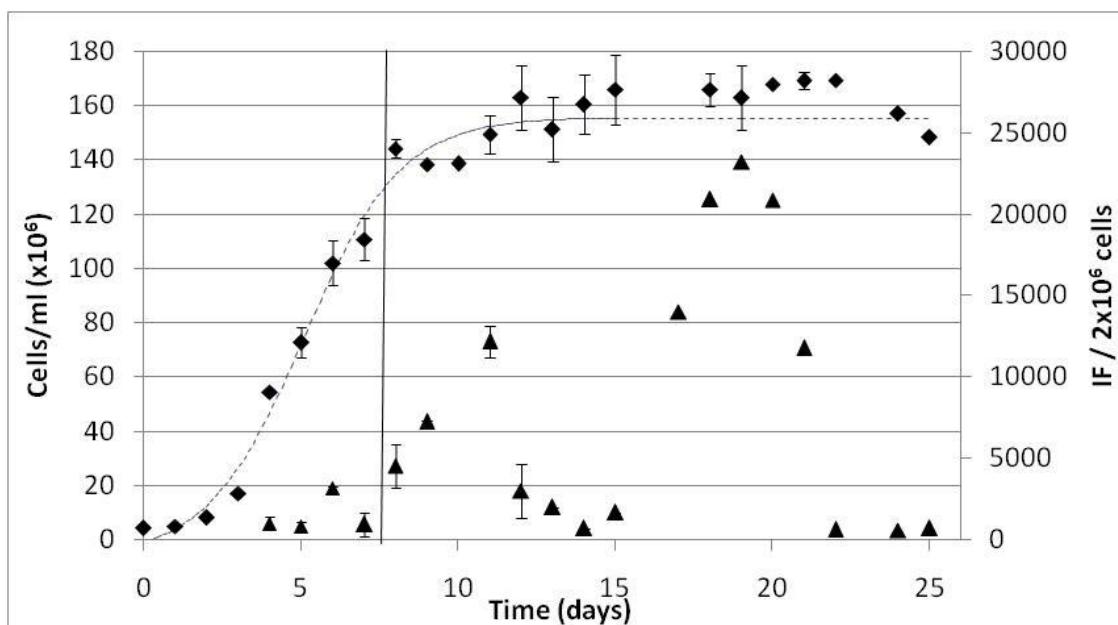


Figure 5.4 The effect of N-limitation on *N. oleabundans* growth (♦) and IF (▲) is reported: the line indicates the centrifugation and resuspension of culture in nitrogen depleted medium.

5.3.4. Effect of temperature on growth

Freshwater species could grow in higher temperature ranges, due to their source. In fact, temperatures are generally higher in freshwater environments than in the sea. So, one of the issues that must be solved at industrial scale, is the control of temperature. In particular, a species tolerant to temperature changes could be interesting from this perspective. To this aim we measured growth curves at 30°C. The two strains of *B. braunii* are not able to grow at that temperature, since the OD do not increase after four days. On the other hand, *N.*

oleabundans, and the *Strain7* and *20*, increased the growth rate if grown at 30°C, as reported in table 5.3, and figures 5.5 and 5.6.

Table 5.3. Specific growth rate (μ) and duplication time of microalgae in bubbling system at 30°C.

Species	μ (days $^{-1}$)	t_d (days)
<i>N. oleoabundans</i> 1185	0.84	0.82
<i>Strain7</i>	0.94	0.74
<i>Strain20</i>	0.99	0.70

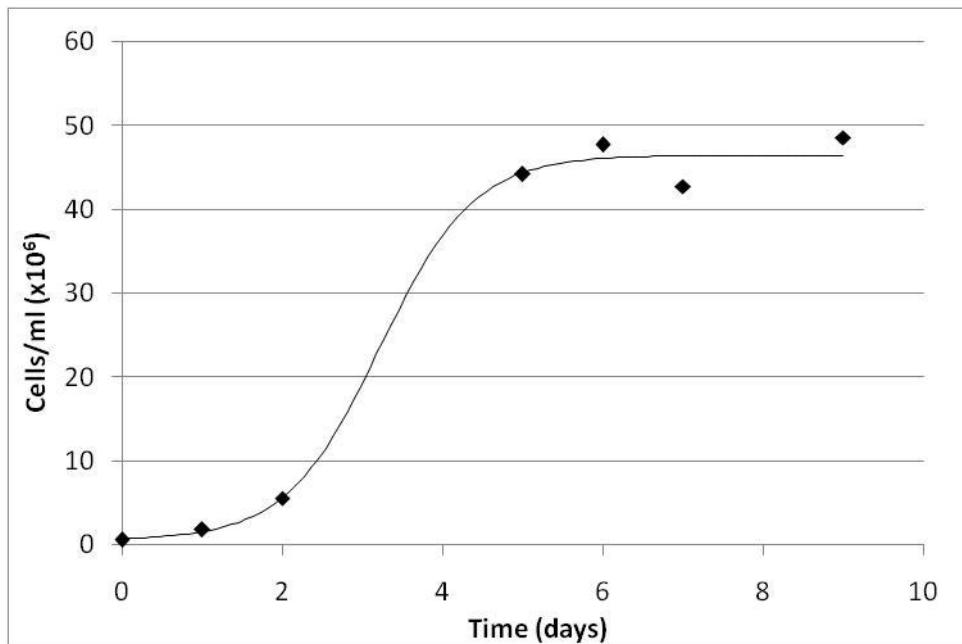


Figure 5.5 Growth curve (♦) of *N. oleoabundans* at 30°C, under autotrophic conditions and minimum media composition. These runs were performed in bubbling tubes.

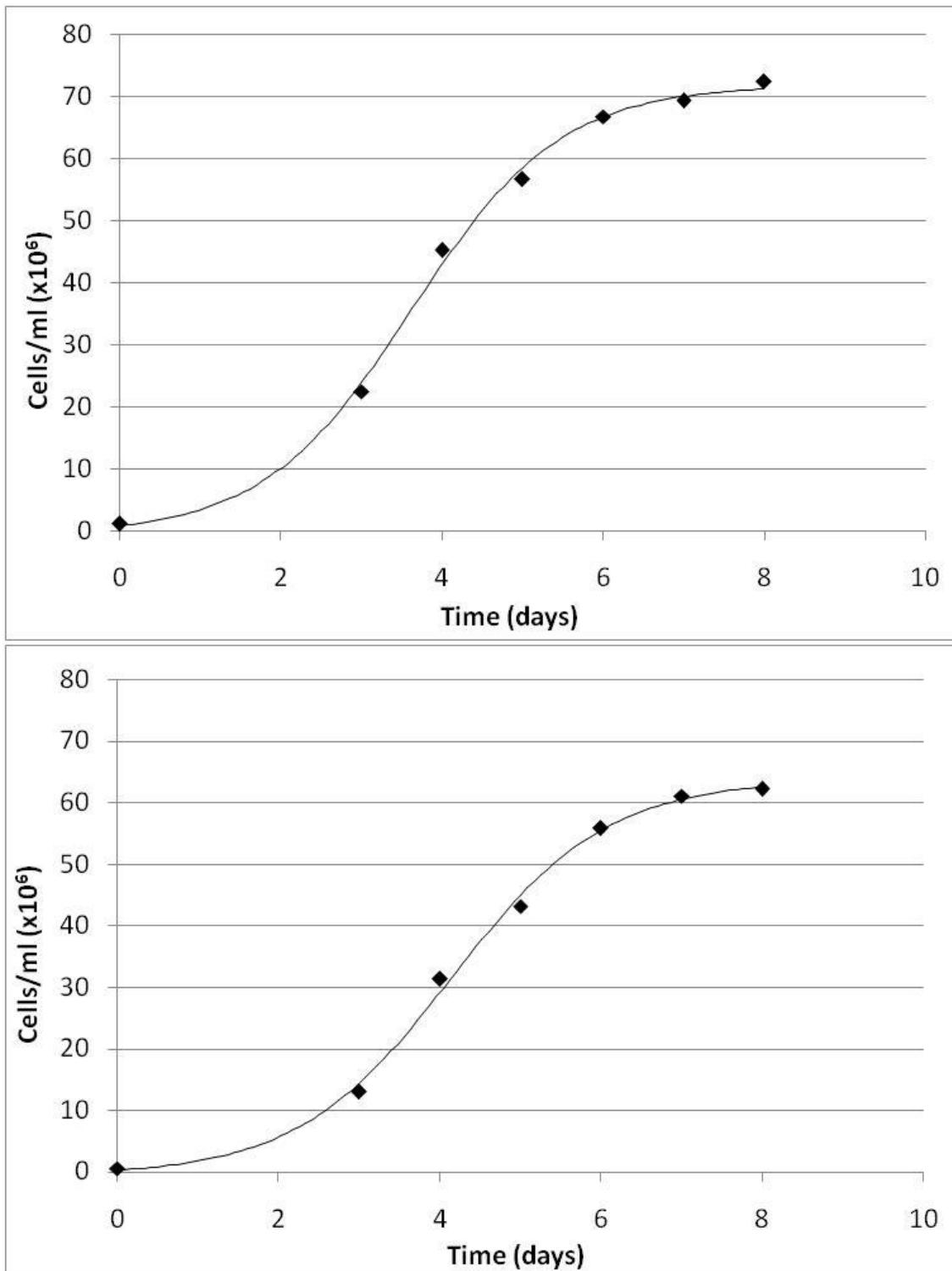


Figure 5.6 Growth curve (♦) of Strain20 (A) and Strain7 (B) at 30°C, under autotrophic conditions and minimum media composition. These runs were performed in bubbling tubes.

5.3.5. Growth curves with industrial byproducts

In an industrial production process, some byproducts can be utilized for feed microalgae in a photobioreactor. In particular, we used a stream of water containing ammonia, and a gaseous stream containing about 10% of CO₂ from a combustion step. This section discusses the possibility of growing algae with these byproducts. The effect of two byproducts were distinctly measured in two different experiments.

- **Process waters as nitrogen source**

The effect of ammonia contained in water from process, was tested at the lab scale, in the bubbling system previous described and with the same medium composition as in table 2.2 except for nitrogen. Thus, we could use the previous experiments as controls and discriminate the effect using this water as a single variable.

It's noteworthy that the nitrogen content in the water (170 mg/Las NH₄⁺) is lower than that contained in culture media used in previous experiments .

Results are reported in table 5.4.

Table 5.4. Specific growth rate (μ) and duplication time of *N. oleoabundans* with industrial by products.

Byproduct	μ (days ⁻¹)	t _d (days)
Process water	0.32	2.16
Combustion gas	0.38	1.82

It was concluded that *N. oleoabundans* is able to use ammonia contained in process waters, as the only source of nitrogen. Anyway, due to the lower content of nitrogen in waters, the growth was slower than in laboratory system, with standard culture media.

- **Combustion gases as carbon source**

In order to measure the growth of microalgae by using CO₂ from combustion gases, containing about 10% (v/v) of CO₂, we set up a basic experimental apparatus on an industrial site. The experimental apparatus is composed by a stream of combustion gases that flows through a condenser: the cooled gas is then delivered to the bubbling system through a silica gel trap, in order to avoid moisture in the stream, and it is pumped to the microalgae suspension. Bottles were under constant photon density of about 100 $\mu\text{Em}^{-2} \text{s}^{-1}$ and at ambient temperature of about 28°C.

According to the results reported in table 5.4 microalgae are able to use CO₂ from combustion gases to grow, although the growth rate is lower than at laboratory conditions. It is noteworthy that the content of CO₂ in gas is very high and the slower kinetic of growth could be due to an inhibitory effect of a consequent pH decrease. To overcome this problem in industrial

applications the gas could be diluted with air, in order to decrease the CO₂ concentration. Anyway, these preliminary results showed that combustion gases is a viable alternative byproduct to feed microalgae and at the same time, a possibility for CO₂ sequestration.

5.3.6. Sedimentation rate of species with different lipid content

Gravity sedimentation is commonly applied for separating microalgae in water and wastewater treatment. Density and radius of algae cells and the induced sedimentation velocity influence the settling characteristic of suspended solids (Chen et al., 2011; Brennan and Owende, 2010). Enhanced microalgal harvesting by sedimentation can be achieved through lamella separators and sedimentation tanks (Uduman et al., 2010). Flocculation is frequently used to increase the efficiency of gravity sedimentation. The success of solids removal by gravity settling depends highly on the density of microalgal particles.

However, the biomass recovery step is the bottleneck of the feasibility of the process, and is important to take it into account moving from lab to pilot scale.

In this work, some preliminary tests of sedimentation rate of microalgal biomass were carried out. In particular, the aim of these experiments is to understand the effect of lipid content on sedimentation rate. Thus, we selected *Strain 7* and *20*, which have different lipid content, but similar characteristics concerning size and biochemical composition of the cell wall. To measure the sedimentation rate, typical protocols from water purification and treatment was used.

In addition, the sedimentation rate was measured at two concentrations of biomass (1g/L DW and 2g/L DW) in a graduated cylinder with a diameter of 5.5 cm. The height of the solid interface in the water column was measured during time and the sedimentation rate was determined as the slope of linear phase of sedimentation curve. Results are reported in table 5.5 and figure 5.7.

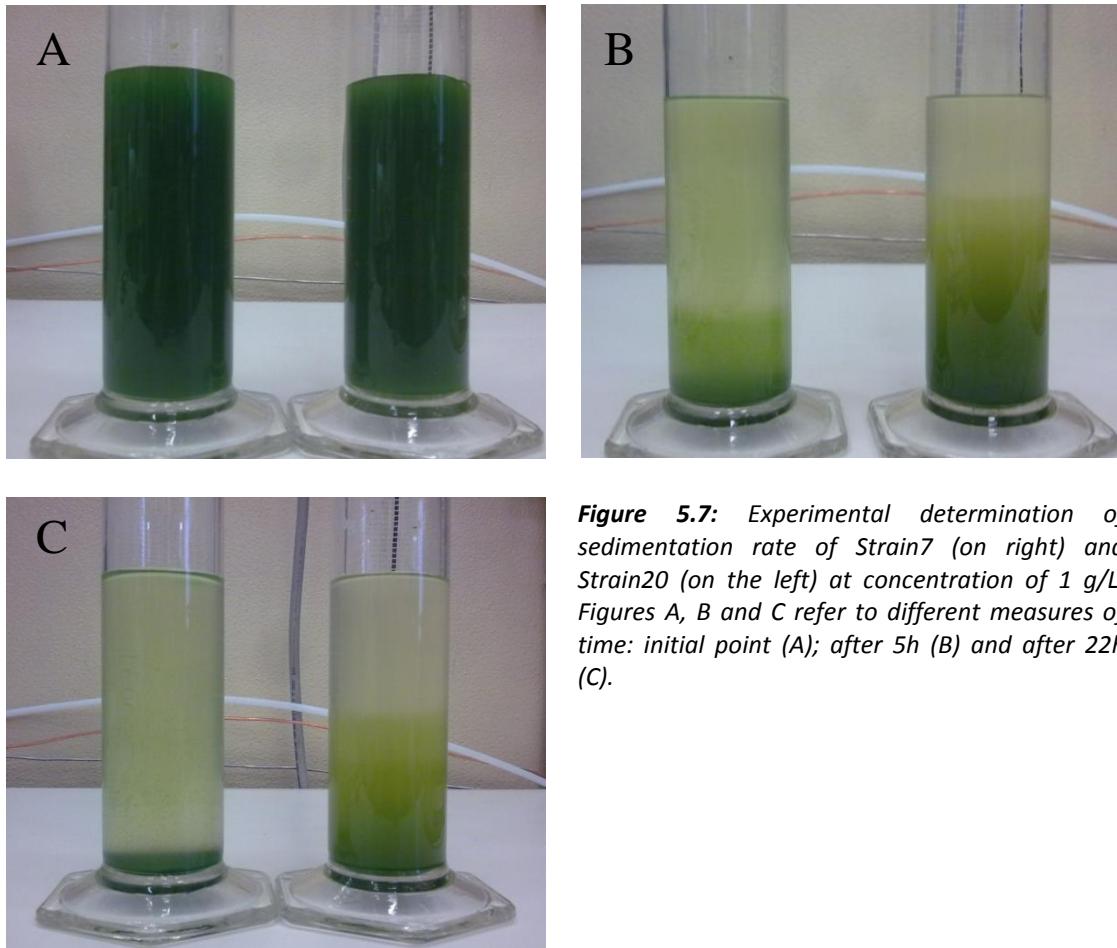


Figure 5.7: Experimental determination of sedimentation rate of Strain7 (on right) and Strain20 (on the left) at concentration of 1 g/L. Figures A, B and C refer to different measures of time: initial point (A); after 5h (B) and after 22h (C).

As reported in figure 5.7 and 5.8 and table 5.5, *Strain7* had a lower sedimentation rate than *Strain20*, probably due to the higher lipid content of the first one: an higher lipid content, corresponding to a lower total density, affected sedimentation rate in water. However the sedimentation rate was low for both strains. It's noteworthy that the diameter of the cylinder could affect the measure, because of the interaction of the solid particles with the walls of the cylinder. This hypothesis is confirmed by the formation of a deep meniscus in the interface. Concerning the effect of the concentration of sedimentation rate, we can state that a lower concentration increased the sedimentation rate. On the other hand, the measures were strongly affected by the optical determination of the interface, which could be inaccurate at lower concentration of biomass.

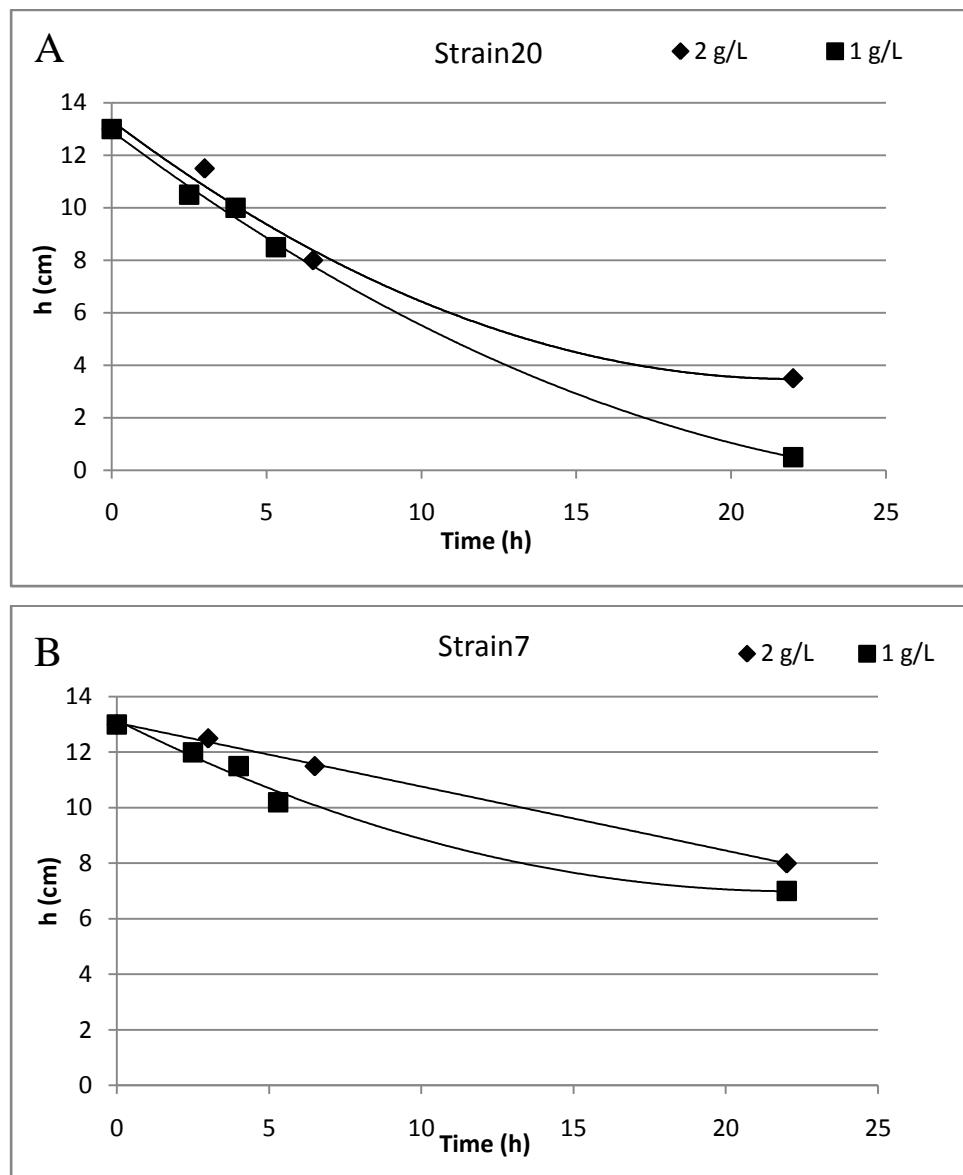


Figure 5.8: Sedimentation rate of *Strain20* (A) e *Strain7* (B) at 1 g/L and 2 g/L.

Table 5.5: Sedimentation rate of *Strain20* and 7 biomass at 1 g/L and 2g/L.

	V_s (1g/L)	V_s (2g/L)
<i>Strain20</i>	0,81 cm/h	0,77 cm/h
<i>Strain7</i>	0,49 cm/h	0,23 cm/h

5.4. Final remarks

In this chapter a fresh water microalgae screening was carried out, in order to select an interesting strain that could be used to exploit CO₂ and nitrogen as nutrients from byproducts of an existing plant. The screening is based on growth kinetics and lipid content. *N. oleabundans* is an interesting species, with high growth rate (0.64 d⁻¹) and lipid content (35%DW), with a calculated theoretical oil productivity of 25 t/ha. In addition, *N. oleabundans* can also grow at higher temperature, and showed a good hardiness to environmental changes, in particular during the onsite experiments. On the other hand, *N. oleabundans* show a particular behavior in accumulating lipids, that could make more complicated the process control, by detecting on line the lipid content and harvest biomass when lipid content is high. Anyway, we can conclude that several species might be used for the industrial purposes, although some issues remain unsolved. In particular, different species, showing different environmental behavior, might need also a specific plant design in order to optimize productivity. In addition, preliminary results shown that the oil content of the species is crucial for an effective recovery of biomass in the downstream process. Thus, from an economical perspective, the downstream process to recover oil from biomass are still very expensive and a decrease of costs is still a challenge, but, culturing microalgae may yield high value commercial products. Sale of these high value products can offset the capital and the operation costs of the process.

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CHAPTER 6

EFFECT OF LIGHT ON GROWTH KINETICS AND LIPID ACCUMULATION OF *Nannochloropsis salina*

In order to develop large scale cultivation systems with sufficient productivity research efforts are still needed to optimize a number of growth parameters. Light has a major relevance for algae: if too low, it limits algae growth while, if in excess, it drives to oxidative stress. Light use efficiency in algae large scale cultivation systems must thus be optimized to achieve a sufficient productivity to make the system economically sustainable. In this chapter the influence of light intensity for growth and lipids productivity of *Nannochloropsis salina* was investigated using a flatbed photobioreactor designed to minimize cells self-shading. The influence of different light intensities was studied using both continuous illumination and alternation of light and dark cycles with different frequencies in order to mimic illumination variations due to mixing.

6.1. Introduction

Photosynthetic organisms are receiving a growing attention for their possible exploitation in the production of biofuels to partially replace fossil fuels (Chisti and Yan, 2011, Dismukes et al. 2008, Hannon et al. 2010, Hu et al. 2008, Malcata, 2011). One of the possibilities currently investigated is to produce biodiesel from oil rich seeds. However this process has little chance to be able to replace a significant fraction of fossil fuels because of limited productivity of seeds per unit area and competition with food production for arable land (Singh et al. 2011). One interesting alternative is to exploit some species of algae which are indeed capable of accumulating large amounts of lipids and thus represents a suitable feedstock for biodiesel production. In this case potential oil productivities are estimated to be ten times higher than crops, making these organisms a highly promising source for biomass production on a long term perspective, although strong research efforts are needed to achieve this potential (Amaro et al. 2011, Chisti and Yan, 2011, Malcata, 2011).

Algae are a group of organisms with a large biological variability (Hannon, M. et al. 2010) and species of the genus *Nannochloropsis* are particularly interesting because of their ability to accumulate large amounts of lipids which can reach concentrations up to 65-70% of total dry weight (Boussiba et al. 1987, Hodgson et al. 1991, Rodolfi et al. 2009). Such a massive accumulation of lipids was shown to be activated during the stationary phase of growth in response to stresses such as nitrogen and/or phosphorous starvation (Gouveia and Oliveira, 2009, Rodolfi et al. 2009, Sforza et al. 2011).

In order to exploit these organisms for large scale biofuels production, it is fundamental to optimize several parameters influencing their productivity. Among others, light is a major factor to be considered since on one side it provides all the energy to support metabolism but on the other hand if present in excess, it drives to the formation of harmful reactive oxygen species (ROS) and oxidative stress (Li et al. 2009). When cells are exposed to illumination, one component of the photosynthetic apparatus, i.e. photosystem II (PSII), is continuously damaged and inactivated driving to photoinhibition (Murata et al. 2007). In order to maintain photosynthetic efficiency, this complex must be continuously repaired by re-synthesis of damaged components, in particular one protein subunit called D1 (Nixon et al. 2010). In addition to this repair mechanism photosynthetic organisms exposed to saturating light also prevent oxidative damages by dissipating energy in excess as heat (Li, Z. et al. 2009). It is noteworthy that both the repair of damaged photosystems and the dissipation of energy reduce the overall light use efficiency and thus should be minimized to attain the highest productivity.

Algae in a photobioreactor (PBR) are inevitably exposed to variable incident light due to diurnal and seasonal differences in irradiation. *Nannochloropsis* species have been shown to be able of growing under a large range of illumination intensity, acclimating to different conditions by optimizing the composition of photosynthetic machinery to irradiation (Boussiba et al. 1987, Pal et al. 2011, Simionato et al. 2011). Among the observed responses to different light intensities, there are the modulation of pigments composition as well as the content of carbon fixating enzymes (Fisher et al. 1996, Simionato et al. 2011).

When algae are grown in photobioreactors conditions are different with respect to the natural environment where they evolved. In PBRs, algal culture has a high optical density, which causes a strongly inhomogeneous light distribution. Because of this, surface-exposed cells absorb most of the light, leaving only a residual part of the radiation for the cells underneath, which thus experience light limited growth. On the contrary, external layers are easily exposed to excess light and they need to dissipate up to 80% of photons as heat to avoid radiation damages, thus strongly reducing light use efficiency. Consistently with this idea, it has been shown that overall efficiency of photobioreactors increase when the light path decreases down to 0.5 cm, thus reducing the in-homogeneity of light distribution (Richmond et al. 2003).

As an additional parameter it should be considered that cells in a photobioreactor are rapidly mixed and abruptly moves from darkness to full sunlight (Carvalho et al. 2011). Such alternation of light and dark periods has been suggested to be beneficial to photosynthetic efficiency (Gordon and Polle, 2007, Kim et al. 2006, Matthijs et al. 1996, Phillips and Myers, 1954, Xue et al. 2011), but it can also lead to stronger light stress (Kulheim et al. 2002).

Mixing cycles of a millisecond time scale (i.e. turbulent eddies) are closer to the photosynthetic turnover time, and consequently could match the energy captured by microalgal antennae during the flashing phase with the rate of carbon fixation that continues during the dark phase, leading to a more efficient use of light (Carvalho et al. 2011). However, cells density plays a crucial role in photosynthetic yield during light / dark cycles (Park and lee 2001): at high concentration, a more intense photosynthetic photon flux helps photons to penetrate deeper into the culture, thus reducing mutual shading, as well as the ratio of cells receiving enough light to perform photosynthesis (Malcata, 2011).

Thus, from an industrial perspective, mixing and the resultant turbulence could increase the photosynthetic efficiency and productivity: much higher rates of turbulence can be achieved in closed tubular reactors, by using higher flowrates in channels reactors, or optical fiber-based PBRs (Carvalho et al. 2011). On the other hand, laminar flow is often a problem in open raceway ponds (Grobbelaar, 2010), affecting the possibility to obtain an efficient mixing. The scaling up of PBRs with higher fluid turbulence is actually a challenge, and a number of papers has already focused on simulating the hydrodynamics in photobioreactor in order to enhance the mixing along the light gradient in the culture, in flat plate or in tubular reactors (Perner-Nochta and Posten, 2007, Yu et al. 2009).

In this chapter we addressed and evaluated the influence of illumination conditions on *Nannochloropsis salina* growth and lipids productivity using a flat-bed photobioreactor designed to minimize self-shading. Algae have been grown under different continuous irradiances but also with dark / light cycles of different intensities and frequencies, to simulate changes in illumination due to mixing in a photobioreactor. Results obtained showed that if mixing is appropriately optimized a photobioreactor might be able to exploit even very intense irradiances with high efficiency. If alternation of dark and light are instead not optimized, a pulsed light can easily drive to growth inhibition.

6.2. Materials and methods

6.2.1. Microalgae and media composition

Nannochloropsis salina 40.85 strains was obtained from SAG-Goettingen and was cultured in sterilized sea salts 22 g/L solution enriched with f/2 Guillard solution as described in section 2.1.1., modified by adding an excess of nitrogen source (1.5 g/L of NaNO₃).

6.2.2. Culture conditions

Growth experiments were performed in a 0.8 cm deep flatbed apparatus (section 2.5.2.). Constant illumination between 5 and 1200 $\mu\text{E m}^{-2}\text{s}^{-1}$ was provided using a LED Light Source SL 3500 (Photon Systems Instruments). Light source was also programmed to generate square-wave dark / light cycles of the desired intensities and frequencies described in figure 6.2. Parameters defining flashes are flash time (t_f), dark time (t_d), duty cycle (ϕ , corresponding to $t_f / (t_f + t_d)$) (Phillips and Myers, 1954, Vejrazka et al. 2011), flash light intensity (I_0) and integrated light intensity (I_a). Algal growth was measured by daily changes in optical density OD₇₅₀ (Lambda Bio 40 UV/VIS Spectrometer, Perkin Elmer) and cells number monitored with a Bürker Counting Chamber (HBG, Germany). The specific growth rate was calculated by the slope of logarithmic phase with respect to the number of cells. All curves were performed in at least four replicates.

6.2.3. Growth analysis and analytical methods

Growth experiments were performed in flat panel reactors under a continuous enriched CO₂ feed flow, as described in section 2.3. Algal growth kinetics were measured by daily changes in optical density and cells number as described in section 2.1.

The lipid content was determined by staining the algal cell suspension with Nile Red (NR) dye as described in section 2.1.

6.2.4. In vivo monitoring of photosynthetic parameters.

Chlorophyll fluorescence was determined in vivo using Dual PAM 100 from WALZ. The parameter Fv/Fm was calculated respectively as (Fm-Fo)/Fo (Demmig-Adams et al. 1996) after 20 minutes of dark adaptation. This parameter allows to quantify the photochemical and non photochemical quenching.

6.2.5. Pigments extraction and analysis.

Chlorophyll a and total Car were extracted from *Nannochloropsis* centrifuged cells with 100 % N,N'-dimethylformamide for at least 48 hours at 4°C in dark conditions according to Moran and Porath (1980). The pigments concentrations were determined spectrophotometrically using specific extinction coefficients (Porra et al. 1989, Wellburn, 1994).

6.3. Results

6.3.1. *Nannochloropsis salina* growth using different illumination intensities.

The influence of light intensity on *Nannochloropsis salina* growth has been assessed in a flatbed photobioreactor designed to reduce self-shading influence on observed growth rates and productivity (figure 2.8). All analyses were performed with low optical density cultures, to further reduce the effect of self-shading on growth kinetics. CO₂ and Nitrogen (as nitrate) were instead provided in strong excess in order to avoid growth limitation due to their scarcity. As shown in Figure 6.1, light strongly influenced both growth rate and final cellular concentration reached by cultures. Between 5 and 150 μE m⁻²s⁻¹ the growth increased with illumination, but over 150 μE m⁻²s⁻¹, where biomass accumulation is maximal, light increase has an inhibitory effect. With the highest intensity tested (1000 μE m⁻²s⁻¹) cultures show a growth similar to that at 350 μE m⁻²s⁻¹, suggesting that cells are able to protect themselves from the strong light excess and still maintain a significant biomass accumulation. Interestingly, when the biomass productivity, expressed as cellular concentration, is normalized to the light intensity two clearly distinct regions are observed. Up to 150 μE m⁻²s⁻¹ this ratio is constant, confirming that, in this range, light is indeed limiting for growth and that irradiation energy is exploited with a comparable efficiency. Over this limit the ratio decreases drastically showing that although cells are still capable of showing substantial growth they use light energy with lower efficiency. The analysis of growth kinetics also showed that under strong illumination (350-1000 μE m⁻²s⁻¹) there is a significant lag phase which is instead not detectable for lower values. Most likely it is needed to give cells the time to respond to the intense illumination before they are able to grow significantly (Figure 6.1A). After few days, in fact, *Nannochloropsis* cells resumed growth ability even in the most extreme conditions.

Data in figure 6.1B display a good correspondence between growing rate and final biomass accumulation, which both show a very similar dependence on illumination intensity. Since growth rate is mostly influenced by the first days of the curve, while biomass concentration depends on when growth ends, some differences were instead expected owing to shading effects, because high light exposed cells could have taken advantage of some shading. On the contrary, our finding supports the previous assumption that in this system shading effects are minimized.

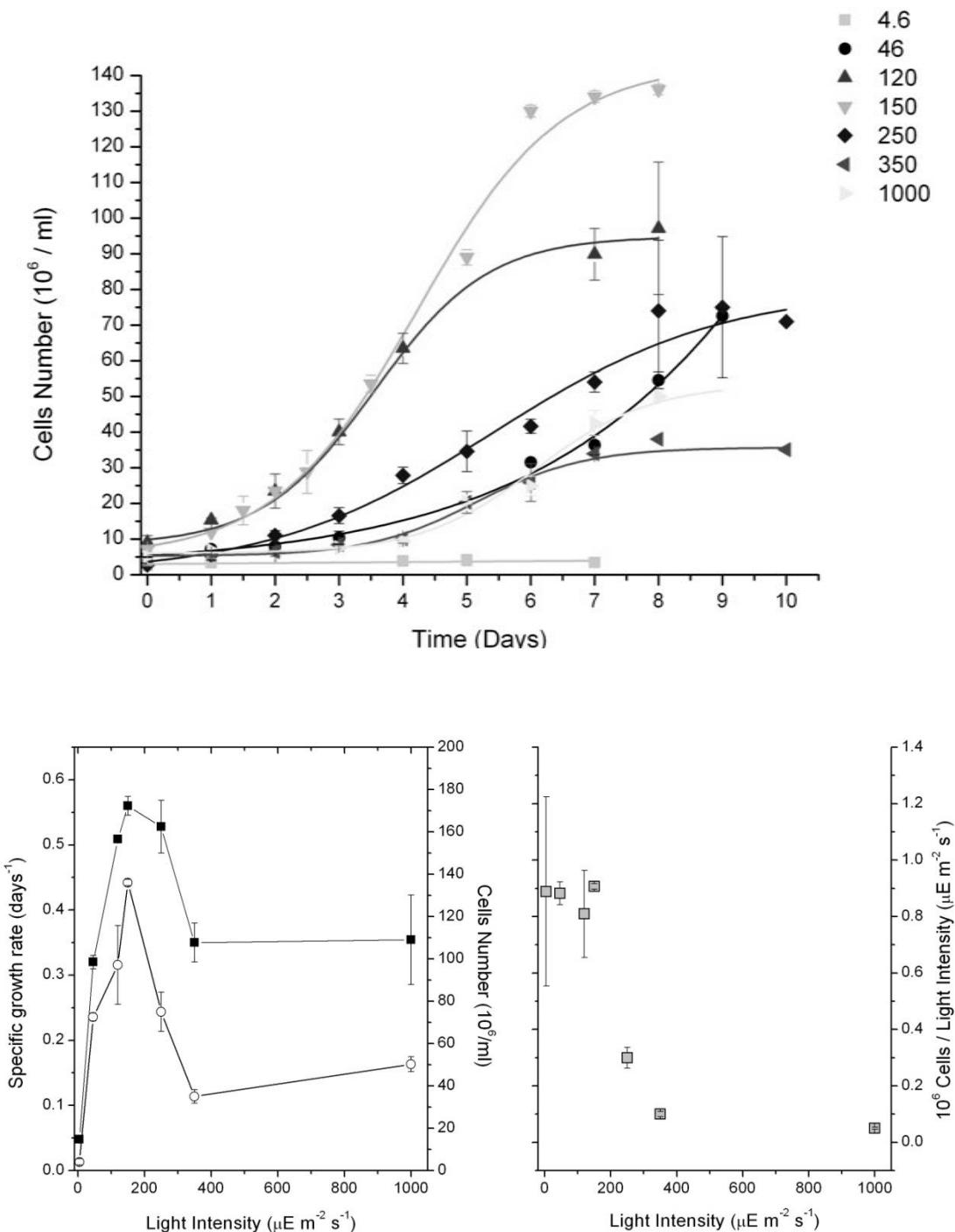


Figure 6.1. *Nannochloropsis salina* growth under different light intensities in a flat bed photobioreactor. A) Growth kinetics of algae exposed to different light intensities ranging from 5 to 1000 $\mu\text{E m}^{-2} \text{s}^{-1}$. B) Growth parameters determined from curves in A, specific growth rate (empty squares) and cellular concentration after 8 days of growth (filled circles). C) Cellular concentrations reported in B normalized to the light intensity.

6.3.2. Effect of pulsed light on *Nannochloropsis salina* growth.

As mentioned above, algae in a photobioreactor are subjected to natural variations in illumination but also to dark/light cycles due to mixing. Accordingly, cells rapidly move from regions where they are fully exposed to sunlight to others where they are shadowed. In order to understand how algae respond to these conditions *Nannochloropsis salina* cells were grown under square-wave light / dark cycles to simulate mixing. All experiments were performed providing a constant total amount of photons, i.e. $120 \mu\text{E m}^{-2}\text{s}^{-1}$ of continuous light. This value was chosen because it represents the highest intensity where the cell growth is still light limited. At fixed integrated light value(I_a), the influence of other parameters, such as frequency and intensity of light pulses was investigated. As reported in table 6.1 and sketched in Figure 6.2, two flashes of different intensities, respectively 350 and $1200 \mu\text{E m}^{-2}\text{s}^{-1}$ were employed. As a result light was supplied for one third and one tenth of the total time (Duty cycles (ϕ) of 0.33 and 0.1), respectively, in order to provide the same total amount of energy. Different frequencies were explored, corresponding to different durations of light and dark phases, as detailed in table 6.1.

Table 6.1. Description of pulsed light conditions employed for *Nannochloropsis salina* growth.

Alternated cycles of light and dark were designed to have all the same integrated light intensity (I_a), corresponding to $120 \mu\text{E m}^{-2}\text{s}^{-1}$ of continuous light. Flashes of two different intensities were employed, 350 and $1200 \mu\text{E}$, with a duty cycle of respectively 0.33 and 0.1. Light changes were performed with different frequencies, respectively 10 and 30 Hz with $350 \mu\text{E}$ and 10, 5 and 1 with $1200 \mu\text{E}$.

Condition	(I_0) ($\mu\text{E m}^{-2}\text{s}^{-1}$)	Frequency of light change (Hz)	Flash time (t_f) ms	Dark time (t_d) ms	(I_a) ($\mu\text{E m}^{-2}\text{s}^{-1}$)	Duty cycle (ϕ)
120	120	-	∞	-	120	1
350-10	350	10	33.33	66.67	120	0.33
350-30	350	30	11	22	120	0.33
1200-10	1200	10	10	90	120	0.1
1200-5	1200	5	20	180	120	0.1
1200-1	1200	1	100	900	120	0.1

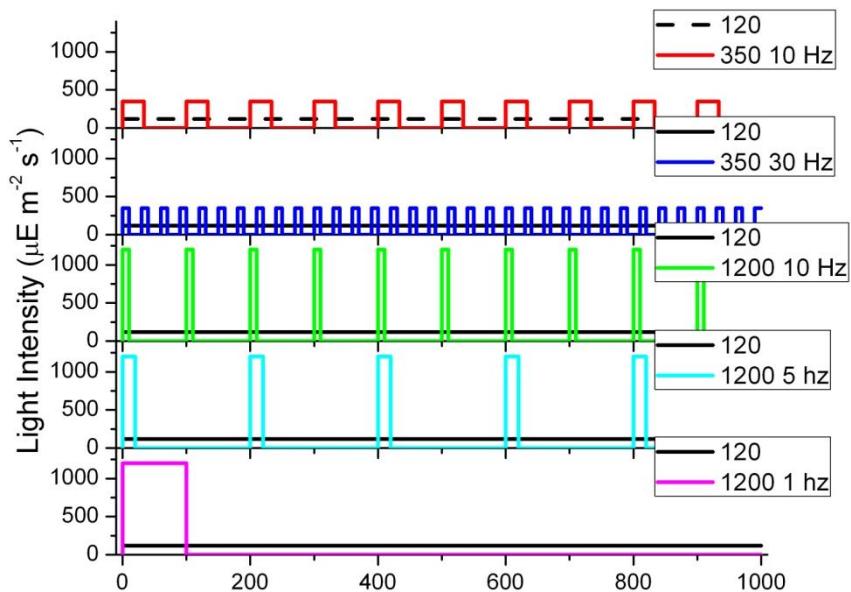


Figure 6.2 Pulsed light conditions employed for *Nannochloropsis salina* growth. Alternated cycles of light and dark were designed to have all the same integrated light intensity (I_a), corresponding to $120 \mu\text{E m}^{-2} \text{s}^{-1}$ of continuous light. Flashes of two different intensities were employed, 350 and $1200 \mu\text{E}$, with a duty cycle of respectively 0.33 and 0.1 . Light changes were performed with different frequencies, respectively 10 and 33 Hz with $350 \mu\text{E}$ and $10, 5$ and 1 with $1200 \mu\text{E}$. These pulsed light conditions resulted in precise durations of flashes (t_f) and dark (t_d), as reported in Table I.

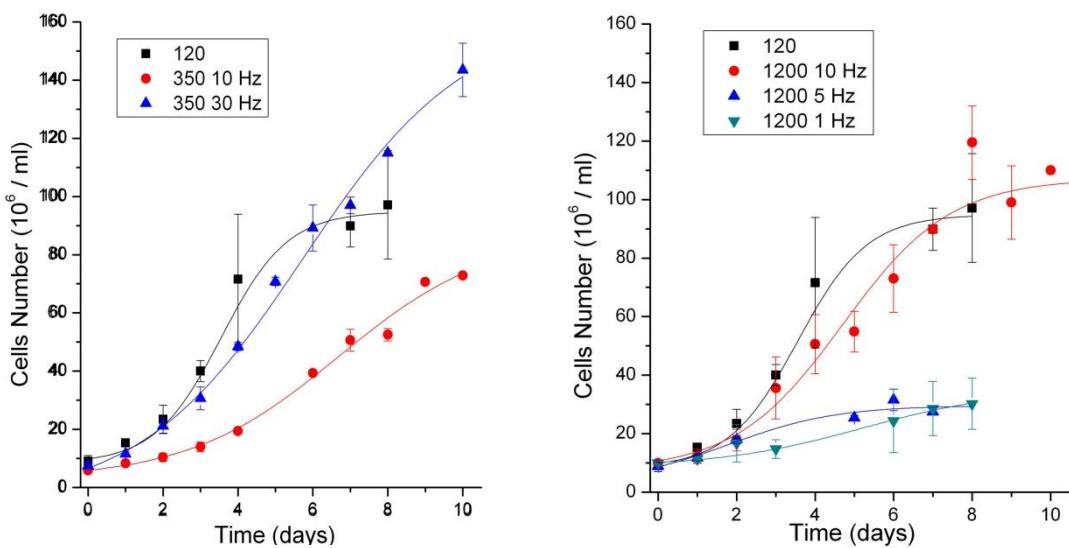


Figure 6.3. Algae growth kinetics under pulsed light. A-B) *Nannochloropsis* growth curves using pulsed light of different intensity and frequencies, $350 \mu\text{E m}^{-2} \text{s}^{-1}$ (10 and 30 Hz, A) and $1200 \mu\text{E m}^{-2} \text{s}^{-1}$ (10, 5, 1 Hz, B). Kinetics at $120 \mu\text{E m}^{-2} \text{s}^{-1}$ continuous light are also reported as comparison (black).

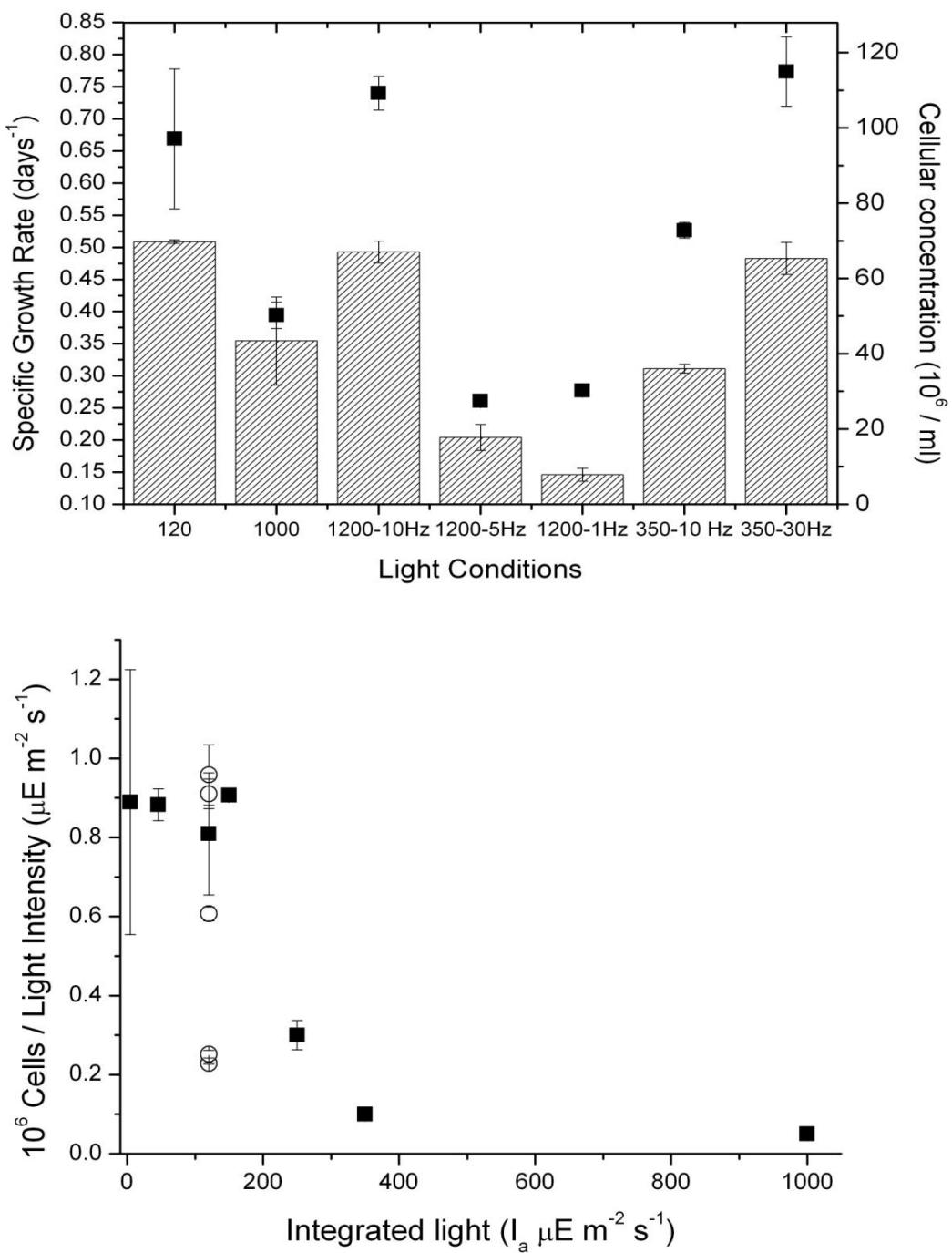


Figure 6.4. Algae growth kinetics under pulsed light A) Growth rate (columns) and final biomass (squares) extrapolated from curves in A-B. Values with 120 and 1000 constant illumination from figure 1 are also reported as comparison. B) Final cell number per light intensity

It was found that using the strongest flashes ($1200 \mu\text{E m}^{-2}\text{s}^{-1}$), the frequency of light changes showed a strong influence on growth. In one case, 10 Hz, growth rate was the same as for the cells exposed to a constant moderate light ($120 \mu\text{E m}^{-2}\text{s}^{-1}$, Figure 6.3A). In these conditions cells showed a complete adaptation, as they grow with alternated light / dark cycles as well as with continuous light (Terry, 1986). If light was provided with lower frequencies pulses, however, such as 5 and 1 Hz, growth was strongly inhibited, even if the total amount of light and the pulses intensities were the same. In the case of 1200-1 Hz and 1200-5 Hz growth was even slower than under a constant and intense light (figure 6.3A-B and 6.4A). If the number of cells per unit of light provided is considered (Figure 6.4B) this clearly shows how 1200-1 and -5 Hz are exploiting light with a very low efficiency.

Similar experiments were also performed using $350 \mu\text{E m}^{-2}\text{s}^{-1}$ flashes. In one case, 350-30 Hz, growth rate and final values were again equivalent to constant $120 \mu\text{E m}^{-2}\text{s}^{-1}$ irradiation, whereas in another one, 350-10 Hz, growth was inhibited by light. The comparison between growth parameters from the two curves (figure 6.4A) showed that frequency *per se* is not a fundamental parameter for determining light influence. On the contrary, curves showing the higher growth ($1200 \mu\text{E m}^{-2}\text{s}^{-1}$ - 10 Hz and $350 \mu\text{E m}^{-2}\text{s}^{-1}$ - 30 Hz) have in common the same length of the illumination phase, which thus has the largest influence on biomass productivity.

6.3.3. Photosynthetic apparatus damages

While influence of pulsed light on photosynthetic efficiency have been already described (Terry, 1986, Vejrazka et al. 2011), the biological effects of these conditions is still obscure. To this aim, photosynthetic properties of the cells grown in different illumination conditions were compared to assess their correlation with growth performances. Fv/Fm is a useful parameter allowing evaluating photosynthetic efficiency in algae and plants and it is particularly helpful in evidencing radiation damages (Maxwell and Johnson, 2000). As shown in Figure 6.5, cells grown with different constant light intensities all show a similar Fv/Fm, around 0.62 ± 0.02 , up to $150 \mu\text{E m}^{-2}\text{s}^{-1}$. Over this limit there is a significant reduction of Fv/Fm value, correlated with the increase of light intensity and consistent with the observation that growth is light inhibited in this conditions. In the pulsed light experiments, Fv/Fm is in the optimal range for both cases where growth rate is also good, 1200-10 Hz and 350-30 Hz. A reduction in Fv/Fm instead observed in all other cases, suggesting that the photosynthetic apparatus in these cells experiences radiation damages. In particular it is noteworthy that in 1200-1 Hz experiment, a reduction in Fv/Fm parameter was found similar to what is observed in the continuous and high light runs, despite now the total energy provided is up to 8.3 times lower. It can be suggested that the abrupt changes in illumination experienced by these cells are as harmful to photosynthetic apparatus as constant high light conditions. In summary, a significant correlation between photosynthetic efficiency and biomass productivity was observed,

suggesting that in the experimental conditions tested the ability of efficiently exploit light energy has the major influence on growth, while the influence of other parameters is minimized.

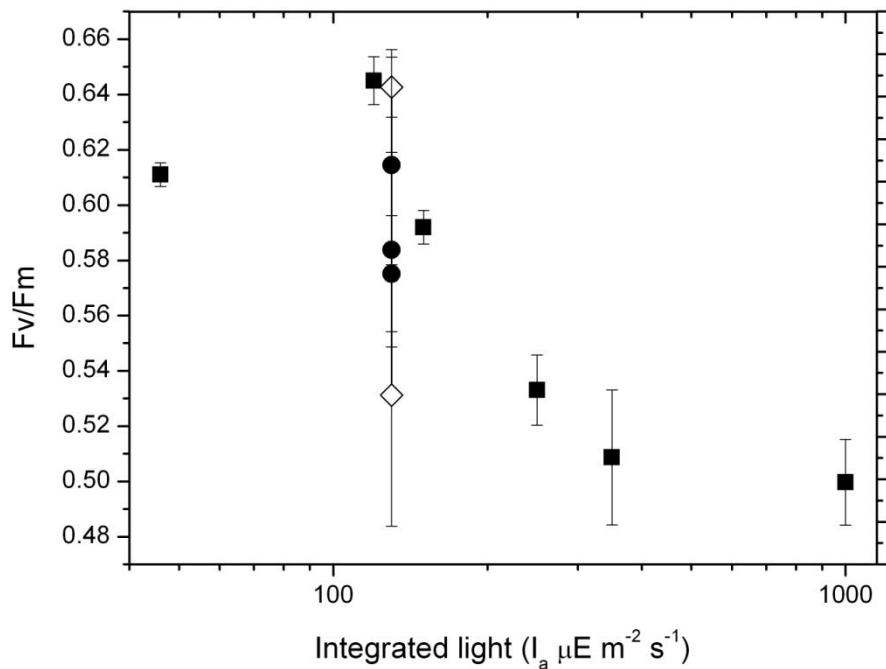


Figure 6.5. Dependence of Photosynthetic Efficiency (F_v/F_m) from illumination conditions. F_v/F_m values at the end of exponential phase is compared between cells grown under continuous illumination of different intensity (black squares) and pulsed light of different intensity and frequencies, $1200 \mu E m^{-2} s^{-1}$ (10, 5, 1 Hz, black circles), $350 \mu E m^{-2} s^{-1}$ (10 and 30 Hz, empty diamonds). Cells grown at $5 \mu E m^{-2} s^{-1}$ were too dilute to provide reliable results.

6.3.4. Acclimation response to alternate light

Nannochloropsis, as many other algae, responds to the different conditions by modulating the composition of its photosynthetic apparatus, a response which is called acclimation (Falkowski and LaRoche, 1991, Simionato et al. 2011). One regulation commonly observed in photosynthetic organisms exposed to different light intensities is the alteration of Chlorophyll (Chl) content per cell and Car/ Chl ratio. In particular under excess illumination, Chl content decreases to reduce the light harvesting efficiency and carotenoids, active in the protection against oxidative stress, are accumulated. Indeed, as shown in Figure 6.6, continuous strong light caused a reduction of Chl content per cells and increase in carotenoids, although differences are smaller than in similar experiments where cells were kept for longer time (6-7 days) in different light conditions (Simionato et al. 2011).

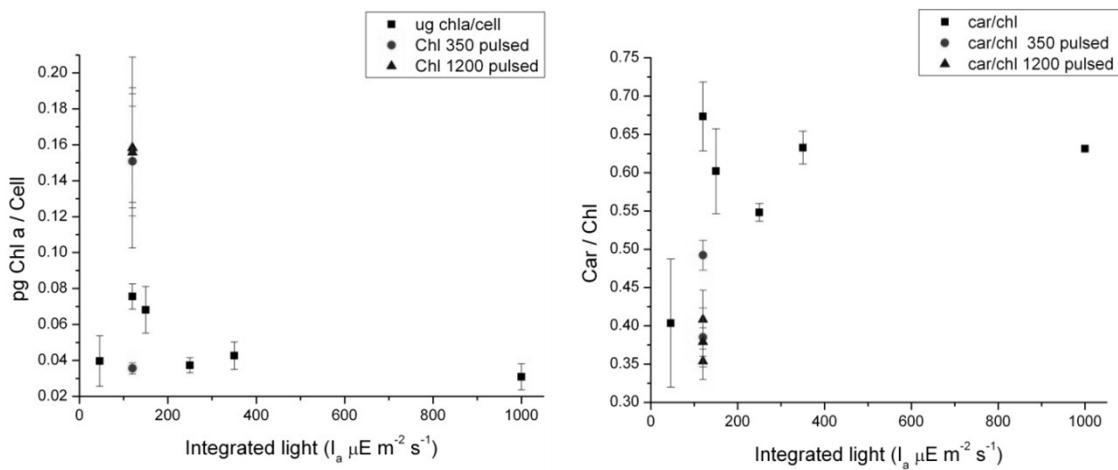


Figure 6.6. Acclimation response in cells grown in continuous vs. pulsed light. Cells grown under different light intensities either continuous or pulsed are compared for their Chl content per cell (A) and Chl / Car ratio (B). These parameters are indicators of the activation of an acclimation response to different illumination.

Despite this reduced time available for acclimation, clear differences were observed for cells under pulsed light: they do not accumulate large amount of carotenoids and even increase their Chl content per cell. These cells showed a response typical of low light adapted cells, with some peculiarities with respect to cultures exposed to the same amount of light intensity, supplied continuously. Furthermore this response did not show to depend on the frequency or duration of light pulses, since similar pigment contents were observed in all conditions but 350-10 Hz, both for cells growing well as for others (1200- 1 and 1200-5 Hz) showing light inhibition. It can be concluded that the presence of a peculiar acclimation response was activated in pulsed light conditions which does not depend on the stress perceived by the cells nor on the total amount of light absorbed.

6.3.5. Accumulation of lipids under high illumination

Light intensity has been suggested to influence algae capacity for lipids accumulation, with strong illumination inducing their accumulation (Damiani et al. 2010). Such a phenomenon has been observed also for species of *Nannochloropsis* group, where a transition from control to high light conditions was found to induce a higher level of lipids accumulation (Fisher et al. 1998, Solovchenko et al. 2010, Sukenik et al. 1989).

The cultures investigated in this work were also evaluated for their lipids productivity. Under low light conditions (up to 150 $\mu\text{E m}^{-2}\text{s}^{-1}$), at the end of the exponential growth phase, cells had a low content of lipids, around 10 % DW, equivalent to the constitutive lipid composing cellular membranes (Su et al. 2011). With irradiances over 150 $\mu\text{E m}^{-2}\text{s}^{-1}$, lipids content reached the maximal accumulation (observed with 350 $\mu\text{E m}^{-2}\text{s}^{-1}$), whit a lipid fraction equal to 70 \pm 9 % of the cells dry weight. When cells were grown in pulsed light conditions, instead, no

induction of lipids accumulation was observed in any conditions tested, independently if cells were growing well or suffered light stress.

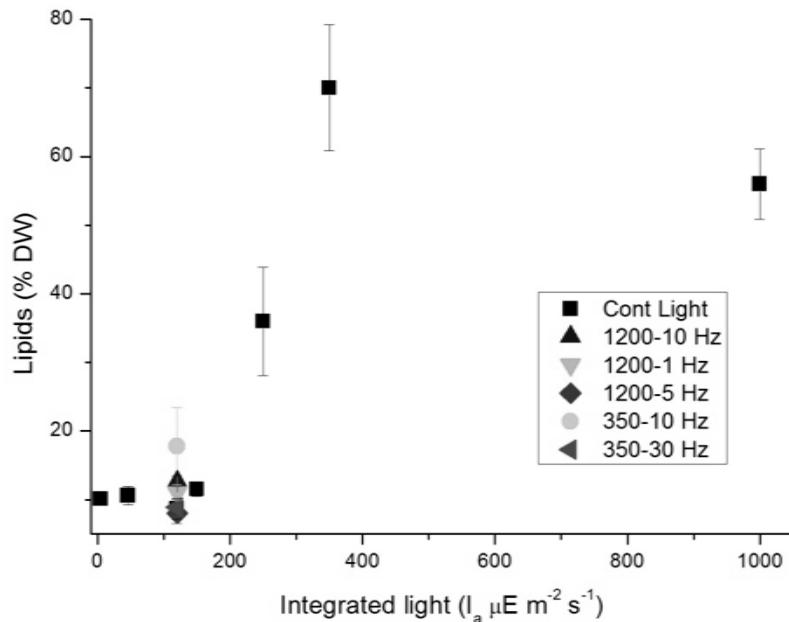


Figure 6.7. Evaluation of lipids productivity. Dependence of lipids production from illumination intensity. Productivity with constant light (black square) are compared with samples with flashes with $350 \mu\text{E}$ and $1200 \mu\text{E}$.

6.4. Discussion and conclusions

6.4.1. Algal growth and lipids accumulation under different light conditions

In this work the effects of light irradiance on *Nannochloropsis* productivity were monitored using a flat-bed reactor designed to reduce as much as possible the effects of cellular self-shading. This configuration is highly useful to investigate directly how illumination influences algae productivity minimizing the shading influence.

By studying the growth rate dependence on intensity of continuous light we observed a first phase when irradiation is limiting and, after a small region of optimal growth, a second one where light has an inhibitory effect. This was expected, considering that light might be limiting for growth but, if in excess, it leads to oxidative stress (Li et al. 2009). The same experiments also showed a strong correlation between photosynthetic efficiency (evaluated from Fv/Fm) and growth performances. In fact, independently on the way light was provided (continuous or with different pulses) when illumination caused light damages it also affected biomass productivity. Thus, in our system, light use efficiency is the major parameter influencing biomass productivity.

When lipids accumulation is considered, the relationship between light intensity and productivity is less straightforward. In fact, experiments with continuous illumination showed

that under increasing light stress a stimulation of lipids accumulation occurs, as suggested by other authors (Fisher et al. 1998, Solovchenko et al. 2010, Sukenik et al. 1989), with 350 μE yielding the best result. It is noteworthy that this lipids production is observed in a medium where nitrogen is provided in excess: thus, it is not achieved by the starvation of this nutrient, as confirmed by the observation that lipids accumulation starts from the early phases of the culture (figure 6.8).

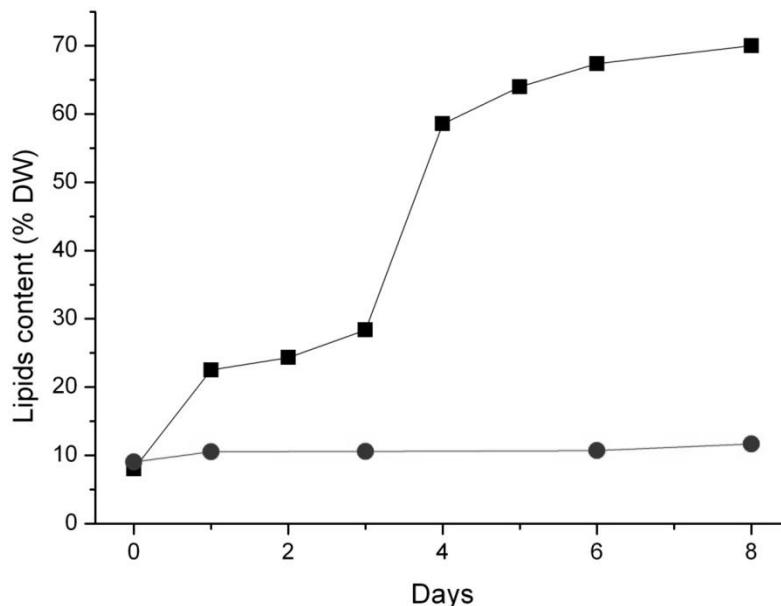


Figure 6.8. Timeline of lipids accumulation in *Nannochloropsis* cells exposed to 350 (black) and 150 (red) $\mu\text{E m}^{-2} \text{s}^{-1}$.

The relationship between light intensity and lipids accumulation is however strongly influenced by other factors. For instance *Nannochloropsis* cells exposed to different radiation intensities in CO_2 limiting conditions showed similar light stress, but this did not influence lipids accumulation (Simionato et al. 2011). This complex relationship was also confirmed by data obtained with cells under pulsed light, here reported. In fact cells exposed to 1200-1 Hz showed light stress and inhibited growth which did not correspond to any lipids accumulation.

6.4.2. Effect of pulsed light – conclusions on mixing

The flat-bed reactor developed and used in this work was designed to investigate how a layer of cells may perform in a larger photobioreactor, where light is in-homogenously distributed and, because of mixing, cells are exposed to abrupt changes in illumination. Experiments with pulsed light of different intensity and duration allowed to assess how these peculiar conditions influence algae productivity. Results showed that, if light is provided with appropriate frequency, even a very strong light, far beyond the saturation point of photosynthesis, can be used to get very efficient growth. For instances cells grew equally well if exposed to $120 \mu\text{E m}^{-2}\text{s}^{-1}$ and 1200-10 Hz, meaning that they are able to use all these photons efficiently (Gordon and Polle, 2007, Matthijs et al. 1996, Phillips and Myers, 1954, Terry, 1986). It is worth underlining that at $120 \mu\text{E m}^{-2}\text{s}^{-1}$ light is still limiting for growth, as shown in figure 6.1A. This means not only that a reduction in light damages occurs but also that light can be harvested and used for growth with the same efficiency as with constant low illumination. Therefore, if dark/light cycles are appropriately set, even strong light intensities over the photosynthesis saturation point could be exploited efficiently. This is possible because photochemical intermediates produced in a short flash of light intensity can be processed further by enzymatic reactions during the following dark period, so that the cell is accomplishing a time dilution of the light energy received.

Similar experiments with different frequencies, however, also showed that alternation of dark/light may be highly dangerous. In fact with 1200-1 and -5 Hz, in fact, light is not exploited efficiently and growth is strongly inhibited. Radiation damages are also identifiable, similar to the ones observed under a constant strong light. Thus, while the alternation of dark light can be helpful, it can also reduce growth efficiency.

Similar results were obtained using light flashes of 1200 and 350 μE , suggesting that the duration of light pulses rather than the frequency of light changes *per se* is the most relevant parameter. This optimal duration was found to be around 10 ms which is consistent with PSII turnover rate in whole cells (Dubinsky et al. 1986, Malcata, 2011). Accordingly, after the photon absorption by the photosystem, 1-15 ms are needed to reset the system, prior of being ready to receive another phonon (Carvalho et al. 2011). If the illumination is this short enough most photons are exploited for photosynthesis and do not drive to the formation of reactive oxygen species (ROS), leading to a photosystems damage. It can be concluded that light is provided for a short time is not harmful. On the contrary it is confirmed that longer times allow generation of reactive species and related damages.

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CHAPTER 7

PRODUCTION OF MICROALGAL BIOMASS IN A CONTINUOUS PHOTOBIOREACTOR

This chapter reports experimental data of microalgae growth and lipid production under autotrophic conditions for the species *Nannochloropsis salina*, in a continuous photobioreactor in order to test the feasibility of the algal biomass production in an industrial continuous process.

7.1. Introduction

Most of the research on microalgal biomass productivity is based on batch lab experiments. Nevertheless, in a batch culture, the cells grow exponentially until limitation, by a nutrient or some other growth factor, or until an inhibitor accumulates sufficiently to prevent further growth. The culture then enters a stationary phase, and eventually a death phase. For large-scale biomass production, continuous or semicontinuous cultures (i.e. chemostat or fed batch reactors) are usually more convenient.

A semicontinuous system is achieved by manually withdrawing a given portion of the culture at periodic intervals (e.g. at the same time each day), and replacing this by pumping in further medium to retain the original volume (Taraldsvik and Myklestad, 2000).

In the continuous flow reactor with perfect mixing (stirred tank reactor, CSTR or chemostat), fresh medium containing growth-limiting nutrients is fed into the bioreactor at a constant rate, and medium mixed with cells is withdrawn from it at the same volumetric rate. A constant bioreactor volume is, thus, maintained and ideally the effluent stream should have the same composition as in the bioreactor. The concentration of the cells in the bioreactor and the cell growth rate (μ) are controlled by the dilution rate (D) or residence time (τ). After a convenient operation time, a steady state cell concentration is reached where the cell density and substrate concentration are constant (Granum and Myklestad, 2002).

One of the most important features of continuous bioreactors is that microalgae can be grown in a physiological steady state. At steady state, growth occurs at a constant rate and all culture parameters remain constant (culture volume, dissolved gas concentration, nutrient and

product concentrations, pH, cell density, etc.). In addition environmental conditions can be easily controlled by the experimenter.

Even though microorganisms are produced most efficiently using continuous cultures many laboratories still use batch or semi-continuous cultivation systems in either carboys or polyethylene bags (Martinez-Jeronimo and Espinosa-Chavez, 1994). The reasons for keep using batch cultures in spite of their inherent limitations are probably larger equipment demands for continuous cultures, increased risks of contamination, equipment failure and wall growth during long cultivation periods, and requirements of skilled labor for maintenance and operation (Eriksen et al., 1998). This may also be the reason of lack of working continuous culture plants. However, in order to achieve a productivity of algal biomass to supply the global energy requirements, a continuous production is required because the effectiveness of continuous cultures is greater than for batch ones. Thus, in this chapter, some basic considerations on massive production of microalgae are described. In addition, some preliminary experiments in semicontinuous systems were carried out, in order to set a continuous experiments of microalgal biomass production using *N. salina* species.

7.2. Biomass production process in continuous system: fundamentals

In order to obtain a continuous production of algal biomass, it is convenient to summarize some considerations on microalgal growth kinetic, residence time and nutrients supply.

7.2.1. Microalgal growth

The Monod kinetics is one of the earliest and most often used equations for describing the relationship between substrate concentration and biomass growth rate. The auto catalytic nature of cell growth leads to a sigmoid growth curve in a batch bioreactor. Monod kinetics generally gives a good fit to the growth curve (Andrea, 1982).

Thus, the growth kinetic of microalgal biomass is formulated as a function of cell and substrates concentrations:

$$R_x = \frac{\mu \cdot c_s \cdot c_x}{K_M + c_s} \quad \left[\frac{g}{L \cdot d} \right] \quad (7.1)$$

where μ is the maximum growth rate for the species considered and c_s and c_x are the concentration of substrate and biomass, respectively. The two constants are the maximum specific growth rate μ , and the saturation constant K_M . If the concentration of substrate in the bioreactor c_s is much greater than the saturation constant then the biomass specific growth rate equals the maximum specific growth rate and therefore the substrate is not rate limiting. On the other hand, if the substrate concentration is less than the saturation constant, then the specific growth rate drops below the maximum and the substrate becomes rate limiting. Thus, the constant K_M is defined as the concentration of substrate at which the growth rate is half of

the maximum value and it accounts for the decrease of the growth rate due to the lack of substrate.

In the exponential phase of growth, nutrients are not limiting and the equation could be expressed as:

$$R_x = \mu \cdot c_x \quad (7.2)$$

where μ is calculated by the slope of logarithmic phase of growth, as also reported in section 2.1.4.

7.2.2. Mass balance of a continuous system

Considering a CSTR model for the biological reactor, macroscopic mass balance of biomass and substrates can be expressed as:

$$c_x^E \cdot \dot{V}^E - c_x^U \cdot \dot{V}^U + R_x^u \cdot V = 0 \quad (7.3)$$

$$c_s^E \cdot \dot{V}^E - c_s^U \cdot \dot{V}^U - \frac{R_x^u}{Y_{x/s}} \cdot V = 0 \quad (7.4)$$

where E and U represent the input and the output, c_x e c_s are biomass and substrate concentrations, \dot{V} is the volumetric flowrate, V the reacting volume and $Y_{x/s}$ the yield of substrate in biomass.

$$Y_{x/s} = \frac{k g_{biomass}}{k g_{substrate}} \quad (7.5)$$

In eq. (7.4) R_x is expressed by equation (7.1). If no biomass is contained in the feed c_x^E is equal to zero: the biomass is exclusively inoculated in the reactor at the beginning. The reactions are carried out in liquid phase, at constant temperature, and the biomass is highly diluted. Consequently, the density of the system could be assumed as constant. If density is constant, the volumetric flow rates in input and output of the reactor are constant, and the mass balances become:

$$-c_x^U + \left(\frac{k \cdot c_s^U \cdot c_x^U}{K_M + c_x^U} \right) \cdot \tau = 0 \quad (7.6)$$

$$c_s^E - c_s^U - \frac{1}{Y_{x/s}} \cdot \left(\frac{k \cdot c_s^U \cdot c_x^U}{K_M + c_s^U} \right) \cdot \tau = 0 \quad (7.7)$$

for biomass and substrate respectively. τ is the residence time of the reactor and is defined as:

$$\tau = \frac{V_R}{\dot{V}} \quad (7.8)$$

Thus, the biomass concentration in the output stream is given by:

$$c_x^U = Y_{x/s} \cdot (c_s^E - c_s^U) \quad (7.9)$$

7.2.3. Flow rate setting

As the biomass concentration in the feed stream is zero, after the initial *inoculum*, the wash out of the system should be avoided, by setting an adequateby small flow rate. This corresponds to a minimum value of τ which is called *wash out* τ :

$$\tau_{wo} = \frac{K_M + c_s^E}{\mu \cdot c_s^E} \quad (7.10)$$

For residence times lower than τ_{wo} the biomass withdrawal is higher than its production. So that the reactor is not able to reach a steady state.

If nutrients are not limiting, the growth kinetics is expressed as eq (7.2) and the constraint on residence time is expressed as:

$$\tau > \tau_{wo} > \frac{1}{\mu} \quad (7.11)$$

In this case the growth rate is directly related to the dilution rate and it must not overcome the wash out of the preinoculated culture. Consequently, the growth rate is affected by D.

In fact, each microalgal species has a maximum specific growth rate (μ), as reported in previous chapters. If a dilution rate is chosen that is higher than μ , the culture will not be able to sustain itself in the bioreactor, and will wash out.

7.2.4. Nutrients supply

Nutrients supply is fundamental to avoid to lower the productivity of the bioreactor. As reported in chapter 3, CO₂, nitrogen and light are nutrients which may largely limit the growth of microalgae.

- **CO₂ supply**

In this section, the effect of CO₂ concentration on growth kinetic is estimated, because the CO₂ concentration in the liquid phase strongly affects the biomass productivity, as reported in chapter 3. In order to calculate the nutrient consumption, the yield of substrate in biomass $Y_{x/s}$, and the elemental composition of microalgae should be considered. Assuming that, among other nutrients, the growth rate is a function of CO₂ concentration only:

$$R_x = \frac{k \cdot c_{CO_2}}{K_M + c_{CO_2}} \cdot Y_{x/s} \cdot (c_{CO_2}^E - c_{CO_2}) \quad (7.12)$$

where $c_{CO_2}^E$ is the saturation value.

As reported in figure 7.1, the growth rate increases when the CO₂ concentration in the reactor increases, because the concentration is rate limiting. At higher concentration the growth rate reaches a maximum, due to biological and physiological constraints.

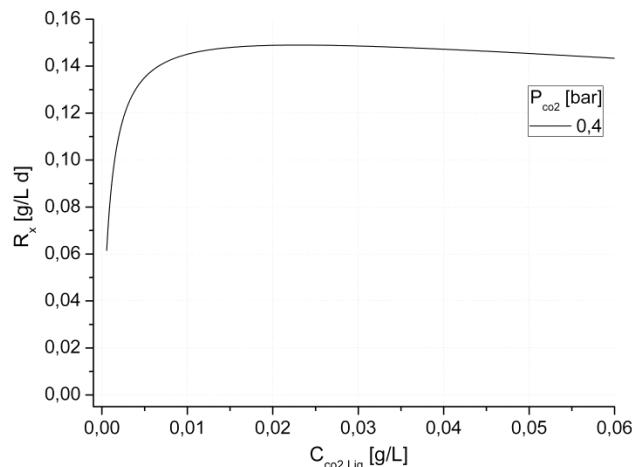


Figure 7.1 Growth rate as function of CO₂ concentration in liquid phase.

Experimental results reported in chapter 3 showed that, for an efficient photobioreactor design, the CO₂ supply should never be limiting during day-light period: such a condition would not only negatively affect the growth rate but also would damage the photosynthetic apparatus with global loss of biomass production.

From an industrial perspective, CO₂ could be derived as byproduct of existing plants. In fact, algae are able to utilize carbon from flue gas emitted by industrial facilities, as demonstrated for *N. oleoabundans* in chapter 5. Overall the CO₂ quantities available from power coal-fired combustion processes are of the order of 15 Gt/y and could substitute more expensive pure CO₂ as a source of carbon for autotrophic growth of algae. Thus, algae for fuel production could be grown using carbon emissions from refineries, power plants and other stationary sources which would otherwise be exhausted into the atmosphere. In fact, CO₂ emissions are normally an unwanted byproduct. Thus, pumping the CO₂ through algae PBRs will reduce the emissions and feed the algae growth leading to faster and cheaper oil production. On the other hand, higher concentration of CO₂ in the bubbling air could lead to a decrease of pH values, negatively affecting algal growth. In order to overcome this problem, the culture medium should be adequately buffered.

- **Light supply**

Microalgae growth also requires an adequate light supply. In autotrophic cultivation, the light is provided by solar radiation. The photosynthetically active radiation (PAR) is about 2700 MJ m⁻² y⁻¹ at the middle latitudes (data from ARPA), and 1 kg of biomass requires about 67 MJ.

Thus, in order to supply a non limiting light radiation, the reactor geometry and the surface exposure play an important role.

Assuming a reactor with a surface S_R and the biomass flowrate W_x , the produced flux of biomass is given by:

$$W''_x = \frac{W_x}{S_R} \quad \left[\frac{g}{m^2 \cdot d} \right] \quad (7.13)$$

Corresponding to an energy needed per surface unit of:

$$E''_x = 67.06 \cdot W''_x \cdot 10^{-3} \quad \left[\frac{MJ}{m^2 \cdot d} \right] \quad (7.14)$$

With PAR accounting for about 40% of incident sunlight on earth's surface, the quantum limit (based on eight photons captured per unit CH₂O produced) on photosynthetic efficiency (PE) works out to roughly 12% (Walker, 2009). Actually, most existing plants fall below this theoretical limit, with global averages estimated typically between 1 and 2% of PE. The reasons for such a difference generally revolve around inefficient uptake of light, or phenomena as photosaturation and photoinhibition. As already reported in chapter 3, a simple calculation of theoretical limit could be done, by taking into account that the maximum theoretical photon conversion efficiency is about 12%. Thus the maximum productivity can be calculated that at best 36 kg m⁻² y⁻¹ of algae (DW), i.e. 360 ton per year per hectare, to fulfill the energy supply constraint. Also in such an ideal condition a photobioreactor autotrophically operated would need huge amounts of light-exposed surface to ensure algae mass production. However, as reported in chapter 6, high intensities could strongly affect the biomass productivity, and geometry and structure of the reactor are the key parameter to avoid photosynthetic damage, leading to unefficient production effectiveness.

Norsker et al. (2011) showed that the cost price of production of algal biomass in a 100 ha plant using flat panel photobioreactors will decrease by 37.5% when the PE increases from 5% to 8%. Different strategies have been adopted to increase the PE in photobioreactors: minimizing the antenna size (Melis, 2009), decreasing the light path of photobioreactors while increasing turbulence in high cell density cultures (Kliphus et al., 2011), and light dilution (Wijffels and Barbosa, 2010, Cuaresma et al., 2011). Increasing PE by genetically engineering algae would theoretically be possible, but this approach still faces regulatory and practical obstacles inside Europe.

Light dilution might be advantageous over increased turbulence since it would not require additional energy input to increase PE (see also chapter 6). The light dilution approach relies on the reduction in light intensity at the reactor surface to minimize the effect of (over)-saturating light conditions. Light dilution by placing the reactor units vertically already proved to increase the photosynthetic efficiency (Cuaresma et al., 2011).

- **Macronutrients supply**

Besides CO₂ and light, algae require nutrients to grow: carbon accounts for an average 45–55% of cell mass, but a typical overall stoichiometry of microalgal biomass includes other components:



as reported by Malcata (2011).

Micronutrients were not considered in this work, because they are commonly present in the coastal sea water. On the other hand, in some cases Fe was found limiting for growth and photosynthetic efficiency in several environments (Morel et al., 1991; Chauhan et al., 2011).

The adequate supply of nutrients is a prerequisite for high production rates: not only the microalgal growth is retarded in the deficient zone, but the deficiency could lead to alien species becoming dominant, increasing infections by bacteria, fungi, viruses, invertebrates and protozoa, and finally to the total collapse of the cultures. Furthermore, microalgae are adapted to scavenge their environments for resources, be it through structural changes, storage or increased resource utilisation efficiencies. Internal adjustments involve biochemical and physiological acclimation, whilst externally they can excrete a variety of compounds to render nutrients available or limit the growth of competitors (Grobbelaar, 2010).

Among macronutrient, nitrogen (N) and phosphorus (P) are the most important ones, appearing mainly in protein, chlorophyll and DNA molecules (Malcata, 2011), with a content of about 3% and 0.3% of dry biomass respectively, based on the calculation of biomass productivity in 1 ha, the nitrogen and phosphorous required as nutrient supply are thus 10 ton y⁻¹ and 1 ton y⁻¹ respectively.

Considering a nitrogen supply as sodium nitrate and phosphorus as sodium phosphate, the corresponding requirements of nutrients are reported in table 7.1.

**Table 7.1 N and P requirements
for 1 ha of photobioreactor**

		ton y ⁻¹
NaNO ₃		61.1
Na ₃ PO ₄		5.1

N and P can be supplied in the form of agricultural fertilizers, which is simple, easily available but can be a significant cost factor (Chisti, 2008) and would require twice the amount of fertilizer that is currently produced in Europe if all transportation fuel were to be replaced by microalgal biodiesel (Malcata, 2011). There are several options for cheaper sources of these nutrients (Aresta et al., 2005). With respect to mentioned wastewater effluents for example

from a fishery, Olguiñ (2010) described a system where 84-96 percent of N and 72-87 percent of P was removed from the anaerobic effluent of piggery wastewater by growing algae, thereby reducing eutrophication in the environment. Another option is nutrient recycling within the process, depending on the treatment technology chosen. For example nutrient recycling after anaerobic digestion or after gasification (Minowa and Sawayama, 1999).

An alternate source of nitrogen supply could be derived from animal farms, that could potentially provide the entire amount required for algal growth (Mata et al., 2010). Similar figures hold for phosphates and trace minerals.

Wastewater from agricultural or domestic sources could be used for nutrient supply, coupling environmental benefit of remediation with an economical sustainability of the biomass production process. For both CO₂ and nitrogen, their concentrations in available waste streams are compatible with those suitable as a feed of an algal photobioreactor. As reported also in chapter 3, species as *N. salina* are able to use nitrogen both as nitrate and ammonia, which is mainly present in wastewaters.

In this section, some preliminary considerations about nitrogen sources are made. For instance, wastewater from anaerobic digestion and biological treatment contains huge amount of nitrogen, that has to be cleaned before discharge in the environment. In table 7.2 contents of nitrogen and phosphorus in typical wastewaters from farming are reported.

Table 7.2. N and P concentration in wastewater from farming.

	Total nitrogen [kg/m ³]	Total phosphorus (P ₂ O ₅) [kg/m ³]
Dairy cattle	2.5 – 3.5	0.8 – 1.5
Meat cattle	3.5 – 4.0	0.8 – 2.5
Calves	1.2 – 3.0	0.6 – 2.5
Swines	1.5 – 3.5	1.1 – 2.7
Poultry and rabbits	1.8 – 14.0	1.5 – 11.0

The surplus of 470 kg ha⁻¹y⁻¹ of nitrogen contributed to environmental pollution by emission of ammonia and nitrous oxide (N₂O) to the air and nitrate to the groundwater. About 50% of the emissions of ammonia and 37% of those of nitrous oxide originated from dairy farming (Aarts et al., 2000).

For instance, a dairy cattle produces an average of 70 kg of N per year. Considering the hypothetical productivity of algal biomass in 1 ha, the requirement of nitrogen is equivalent to that produced by 144 cattle. Thus, deriving nitrogen from wastewaters could be economically effective.

The removal of nitrogen from wastewaters is usually done through the biological oxidation of nitrogen from ammonia to nitrate (nitrification), followed by denitrification, i.e. the reduction of nitrate to gaseous nitrogen, which is released to the atmosphere and thus removed from the water. Nitrification itself is an aerobic process in two steps, each one facilitated by a different type of bacteria. The oxidation of ammonia (NH_3) to nitrite (NO_2^-) is most often performed by *Nitrosomonas* spp. Nitrite oxidation to nitrate (NO_3^-), though traditionally believed to be facilitated by *Nitrobacter* spp., is now known to be facilitated in the environment almost exclusively by *Nitrospira* spp (Tchobanoglou et al., 2003).

After biological treatment, the ammonium concentration is about 1 kg/m^3 , and by calculating the requirement of 1 ha of photobioreactor, the amount needed is 12857 kg/y of ammonium corresponding to a flowrate of $3 \text{ m}^3/\text{h}$, which represents a fraction of those produced in a farming depuration plant. Other sources of nitrogen could be derived by stripping and absorption of ammonia from wastewaters.

In the previous cases, the photobioreactor should be located near to the treatment plant in order to avoid cost of transportation, that may become relevant. In fact, simple calculations demonstrated that, in some cases, if considering the transportation of nutrients, the cost of ammonium sulfate for industrial use could be comparable.

7.2.5. Temperature effect

Microalgae, as other living organisms, need an optimal temperature control in order to avoid lower growth rate or cell death, due to excessive temperature increases. The temperature control in a photobioreactor is a critical point, in particular in temperate and subtropical regions: usually, in that regions, algae have a growth season, so that in winter the outside temperature drops and algae only grow at a fraction of the summertime growth rate. Thus, if a continuous production is required, a cooler system during summer and an heating system in the winter are unavoidable. In many industrial processes, heat is produced. Sometimes this heat is used somewhere else in the process, sold to neighbouring industries or used to heat houses or other buildings nearby. But often this surplus heat has no further use, so that it could be employed to optimize the temperature of the medium in which the algae grow. Especially interesting is the combination with CO_2 of power plants, since power plants are important producers of this surplus heat. On the other hand, closed systems can get too hot and require cooling, which can be achieved by refrigeration with heat exchangers or by spraying water on the outside (Chisti, 2007).

7.3. Materials and Methods

7.3.1. Microalgae and media composition

Nannochloropsis salina 40.85 strains was obtained from SAG-Goettingen and was cultured in sterilized sea salts 22 g/L solution enriched with f/2 Guillard solution as described in section 2.1.1., modified by adding an excess of nitrogen source (1.5 g/L of NaNO₃).

7.3.2. Growth analysis.

Growth experiments were performed both in 0.25-L glass bottles and in flat panel reactor under a continuous enriched CO₂ feed flow, as described in section 2.3. Algal growth kinetics were measured by daily changes in optical density and cells number as previously described in section 2.1.

7.3.3. Analytical methods.

The lipid content was determined by staining the algal cell suspension with Nile Red (NR) dye as previously described in section 2.1. The photosynthetic efficiency and parameters were measured as reported in section 6.2.4.

7.3.4. Equipment

For continuous experiments, a flat panel system was set up (figure 7.2 and 7.3). The flat-plate photobioreactors built and used in this research are made of transparent materials (polycarbonate) for maximum utilization of light energy, as shown in figure.

The working volume is 250 ml and the culture mixing is provided by a air-CO₂ flow through a sparger (described in section 2.2.2) placed in the bottom of the panel. The gas flow also supplies a non limiting CO₂ amount to the culture. The air entering the reactor is regulated using suitable valves and flow meters. The fresh medium is fed at a constant rate by a peristaltic pump (Watson-Marlow sci400, flow rate range: 25-250 ml days⁻¹), and medium mixed with cells leaves the bioreactor at the same rate by an overflow tube and is collected in a tank. The illumination is provided by fluorescent lamps at 120 µE m⁻² s⁻¹.

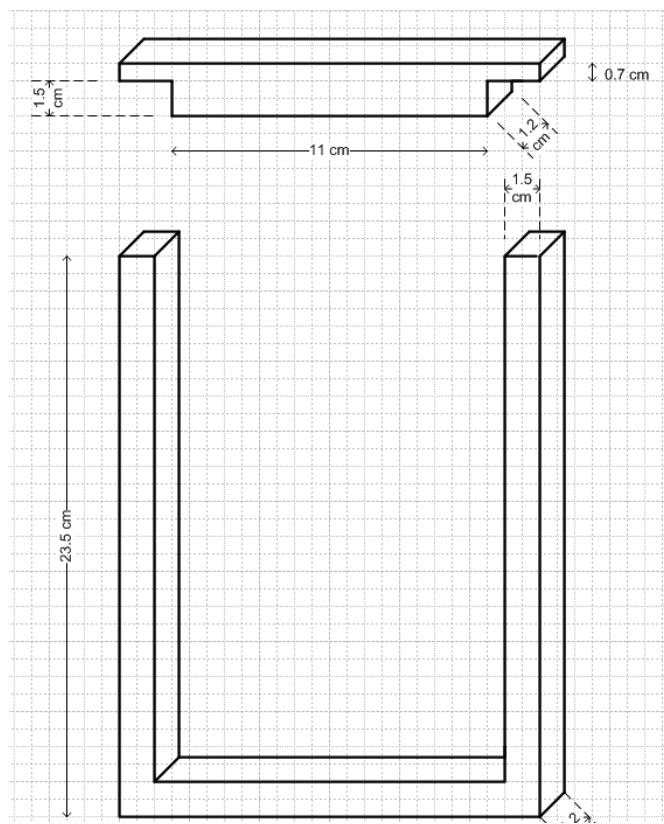


Figure 7.2 Size of the flat panel.

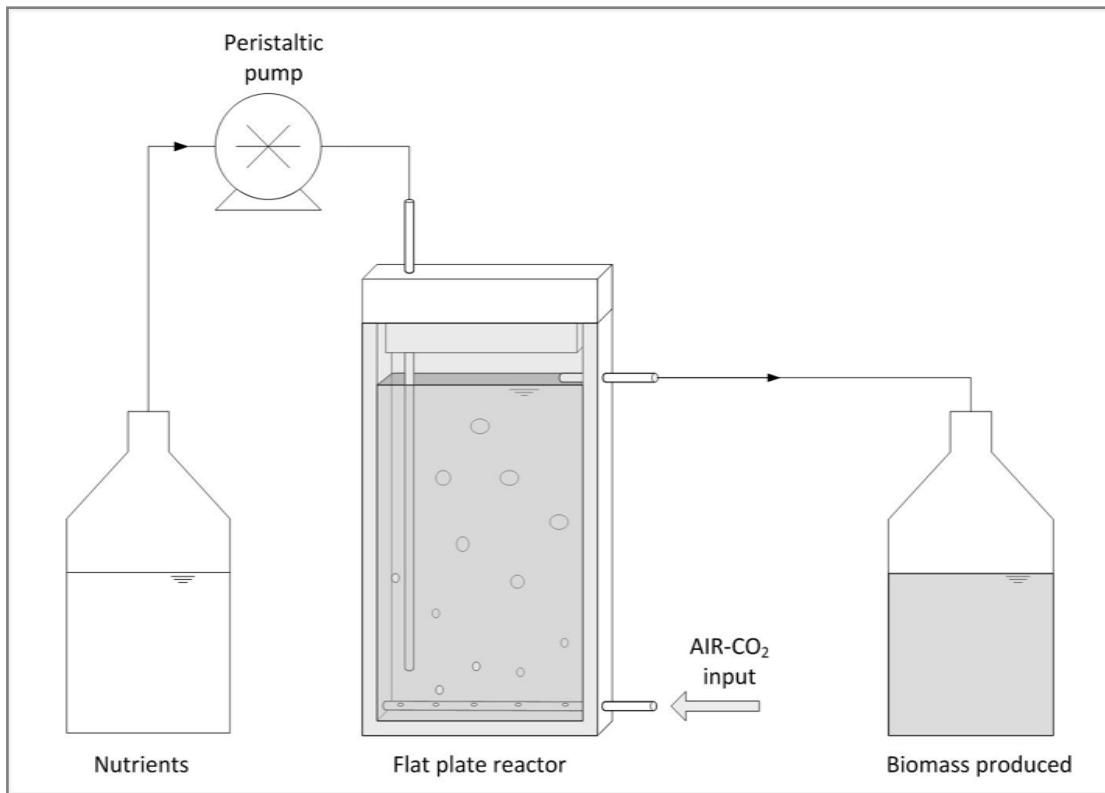


Figure 7.3 Configuration of the continuous reactor system

7.4. Results

In order to understand the feasibility of a continuous production of microalgal biomass, some preliminary test were carried out.

7.4.1. Semicontinuous experiments

A first semicontinuous experiment was performed to test the reproducibility of the growth kinetic in a multiple step process. After an initial growth in batch mode, the culture volume removed was then replaced with fresh medium each day, in order to obtain the same starting concentration of biomass. The experiment was performed for 5 days. Thus, in the semicontinuous culture system, *N. salina* cells were sampled at the time before culture replaced each day for determination of biomass concentration and calculation of specific growth rate. Figure 7.3 summarizes the results: the culture was still growing after each resuspension step in fresh media, with similar specific growth rates. The differences among the growth rates are due to the experimental error on the cell concentration measure.

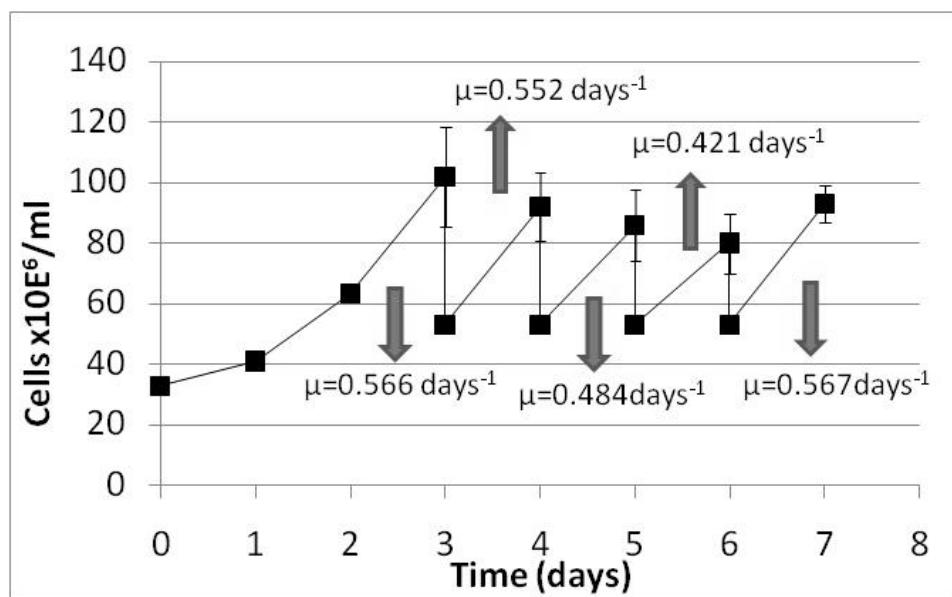


Fig. 7.3. Semicontinuous culture of *N. salina*.

7.4.2. Biomass concentration in stationary phase of a batch growth curve

Microalgal growth in batch culture can be modeled as bacterial growth, with four different phases: lag phase, exponential, stationary phase and death phase, as already shown in previous chapters. During stationary phase the growth rate slows and the biomass concentration remains constant, because the rate of microalgal growth is equal to the rate of microalgal death. The physiological reason of the stationary phase is a result of nutrient depletion or accumulation of toxic products. From an industrial point of view, the maximum biomass concentration strongly affect productivity and biomass recovery efficiency. In particular, a continuous production could be strongly affected if the stationary concentration

is due to an accumulation of inhibitory metabolites. To this aim, some experiments were carried out, in order to verify the cause of the maximum concentration reached by the culture during the stationary phase.

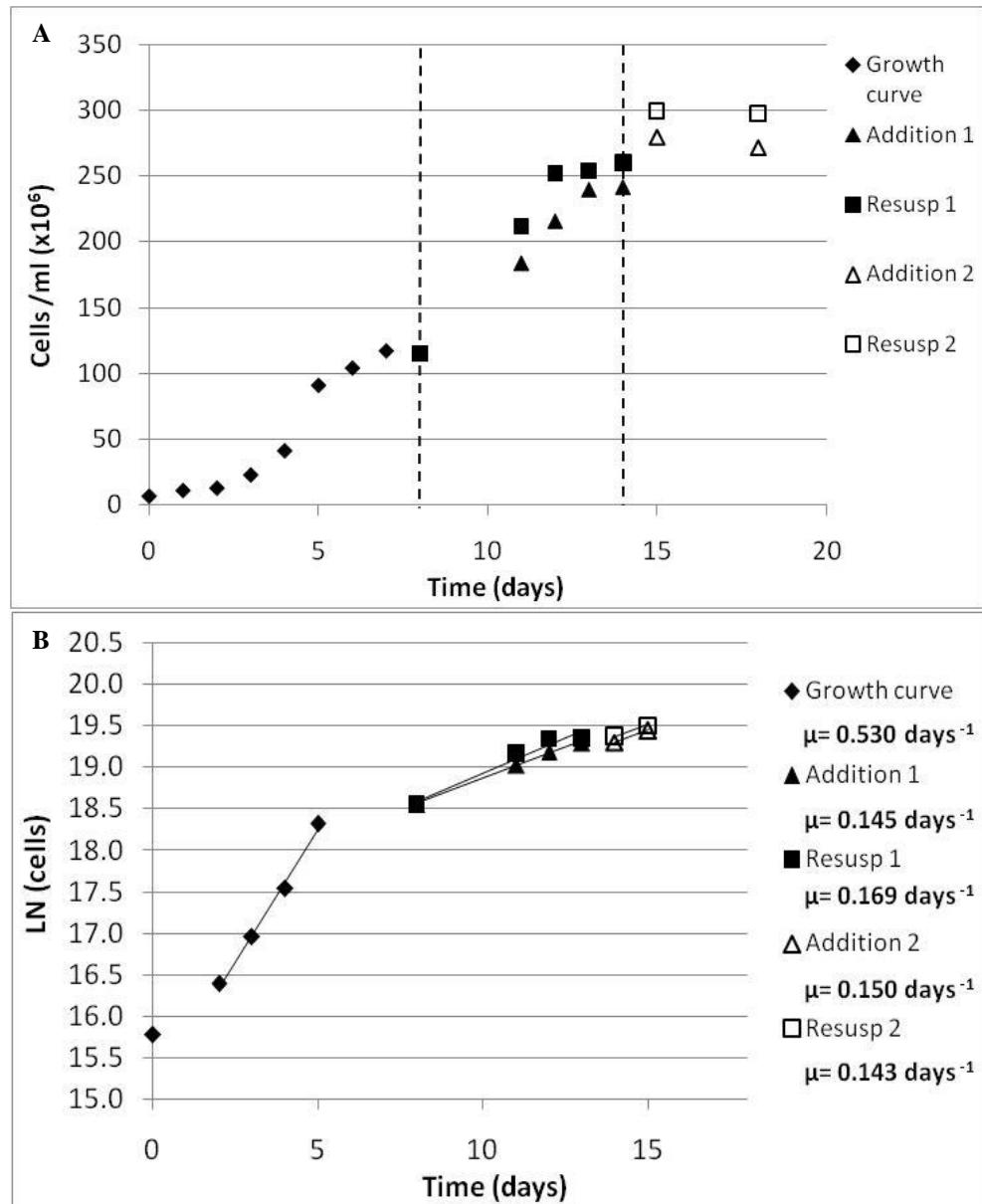


Figure 7.4 Growth curves (fig 7.4A) and semilog graphs (fig 7.4 B) with addition of nutrients or resuspension in fresh media after stationary phase.

As reported in figure 7.4, a *N. Salina* microalgal culture was collected after reaching the stationary phase. A part of these culture was centrifuged and resuspended in fresh medium. As control, in a part of the same culture, nutrients at starting concentration were added. As reported in fig 7.4, after these steps the cell concentration increased again, showing that the stationary phase is merely due to a nutrient limitation. We repeated this operation twice,

obtaining in both cases an increased biomass concentration. Very similar results were found with the two different treatments (resuspension after centrifugation vs nutrient addition), suggesting that no inhibitors accumulation in the suspension occurred.

It is noteworthy that after both resuspension and addition, the growth rate is slower than before. This result is probably due to the quantity of light energy absorbed, which is related to the self-shading of high cell concentration. Thus, all photons of light flux were captured by superficial layers of a high cell concentration culture, and the photons could not pass through a culture. Thus, cell concentration of a photosynthetic culture will continue to increase exponentially until all photosynthetically available photons impinging on the culture surface are absorbed. After that, the growth rate will decrease and the stationary phase will be achieved. These results confirmed the crucial role of the light in a high density culture in view of a large scale biomass production.

7.4.3. Continuous experiment

After these preliminary results, an experiments in continuous mode of operation was carried out, using the flat plate reactor described in section 7.2. The irradiance delivered to the panel was selected as $120 \mu\text{E m}^{-2} \text{s}^{-1}$, on the base of results reported in section 6.4, which demonstrated a non saturating irradiance.

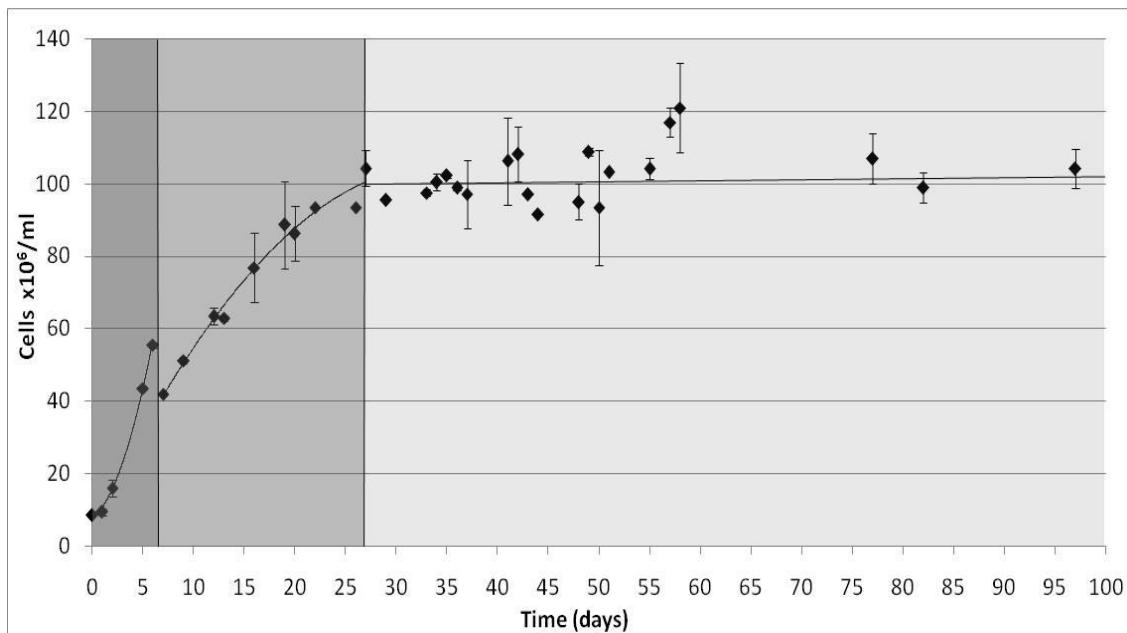


Figure 7.5 *N. salina* cell concentration in continuous flat plate reactor.

The nutrient inlet pump was set at constant flow rate. A peristaltic pump is most suitable for delivery of medium into the culture, as its mechanical parts are not in direct contact with the medium.

The steady-state system was run under continuous illumination to keep a constant cell density, chemical composition and physiological state. The experiment was started in batch mode (see figure 7.5), in order to achieve an higher biomass concentration. On the 7th days, the peristaltic pumps were turned on, and, after 10 days of transient operation, a steady state was eventually reached, with a biomass concentration of about 100 millions of cell/ml, corresponding to about 1.2 g/L DW.

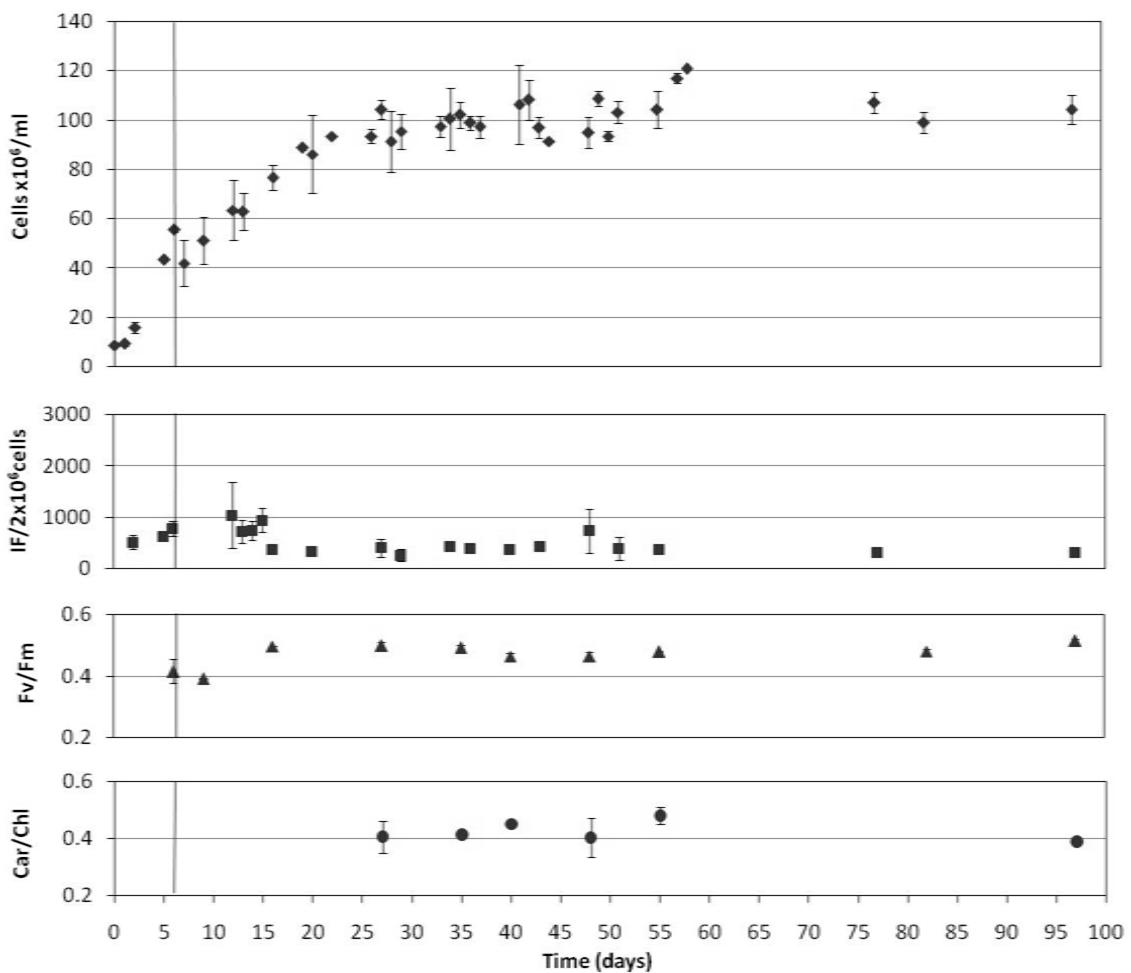


Figure 7.6 Lipid content (fig 7.5B) and photosynthetic efficiency (fig. 7.5C) and pigment content ratio (fig 7.6D) of *N. salina* culture in continuous reactor (fig 7.5A).

The total duration of the experiment was of 100 days, with a constant production of biomass at constant physiological parameters. The flowrate of the pump was set at 76 ml days⁻¹, calculated on the specific growth rate of the data collected during the batch exponential phase of growth, in order to avoid the wash out. Thus, the residence time was 3.24 days and the dilution rate was 0.31 days⁻¹. Accordingly, the biomass productivity was calculated as 0.3 g l⁻¹ day⁻¹ on DW basis.

The steady-state hypothesis seems to be also supported by stability of growth parameters (fig. 7.6), limited cell deposition on the reactor bottom and no cell lysis. Biomass of uniform and controlled amount in terms of oil content was produced: the lipid content of the culture, reported in figure 7.6, remained constant during all the experiment. However, the IF data measured corresponded to a low lipid content, because nutrients were provided in excess and microalgal cells were not stressed. This was also confirmed by the Fv/Fm measurement, which was constant during the experiment, suggesting that the culture is adapted to the irradiation conditions: the PSII efficiency is similar to values measured in batch under the same conditions, in the exponential phase of growth. Concerning the photosynthetic parameters, also the pigment content (Chla content and Chl/Car ratio) remain constant during the continuous experiments, confirming efficiency of the photosynthetic apparatus.

By calculating the specific growth rate from the dilution rate, it seems that the growth rate is lower than registered in batch experiments (0.31 instead of 0.59 days⁻¹). This is probably due to the high cell concentration, causing a shading effect of the impinging light to the surface of reactor. In fact, when measuring by a radiometer the light intensity in front and behind the panel, we found that about 77% of light was absorbed (or dispersed) by the culture although the small depth of the photobioreactor.

In a light limited continuous flow culture, where all incident photosynthetically available radiance is absorbed, the energy balance in the culture could be expressed as follows:

$$\text{Net increase in energy content} = \text{Energy absorbed by biomass}$$

By considering the exposure surface of the reactor (measured as about 210 cm²) and the energy provided to the culture, a biomass productivity of 4.34 g m⁻² days⁻¹ was calculated, corresponding to about 16 ton ha⁻¹ year⁻¹ (under continuous illumination, table 7.3), with a conversion efficiency if 3.8% of PAR (1.63% of total light, considering PAR as 43%).

Tab 7.3 Calculations of energy conversion efficiency in the continuous flat plate reactor

Flowrate	76	ml days ⁻¹
Biomass concentration	1.2	g l ⁻¹
Biomass produced	912	mg days ⁻¹
Exposure surface	210	cm ²
Productivity	4.34	g m ⁻² days ⁻¹
Irradiation	120	μE m ⁻² sec ⁻¹
Average energy of PAR photon	217	KJ mol ⁻¹
Energy supplied to the panel	0.55	J sec ⁻¹
Energy content of biomass	20	MJ kg ⁻¹
Energy fixed by biomass	1.82	KJ days ⁻¹
Energy conversion	3.86	%

7.4.4. A two step process to increase the lipid content

As supposed in chapter 2, species that need a stress to accumulated lipid could be produced only in two step processes, if the final product desired is the oil. This is the case of *N. salina*, that under continuous non limiting production conditions, shown a constant low production of lipids (fig 7.6). Thus, in order to induce lipid accumulation, a second step under stressing conditions should be set. As reported in chapter 3, the nitrogen starvation is responsible to a lipid accumulation. However, other stressing conditions could also trigger this process. In the previous chapter, for instance, we demonstrated that light at high irradiances is responsible of a lipid accumulation. Thus, we measured the lipid content of a culture collected from the output stream of the continuous reactor, exposed at 1200 mEm⁻² s⁻¹, in flat panels bubbled with CO₂ 5%. Results are reported in figure 7.7. In this batch experiments, the cell concentration remained constant, because the light conditions is saturating, but the lipid content increased up to 40% DW. This was also confirmed by the colour of the culture, that shifted from dark green to orange-yellow, confirmed by quantification of the pigments, with an increased car/chl ratio. Actually, there is not a carotenoids accumulation, but a decreased chl content, due to the inhibition by high light.

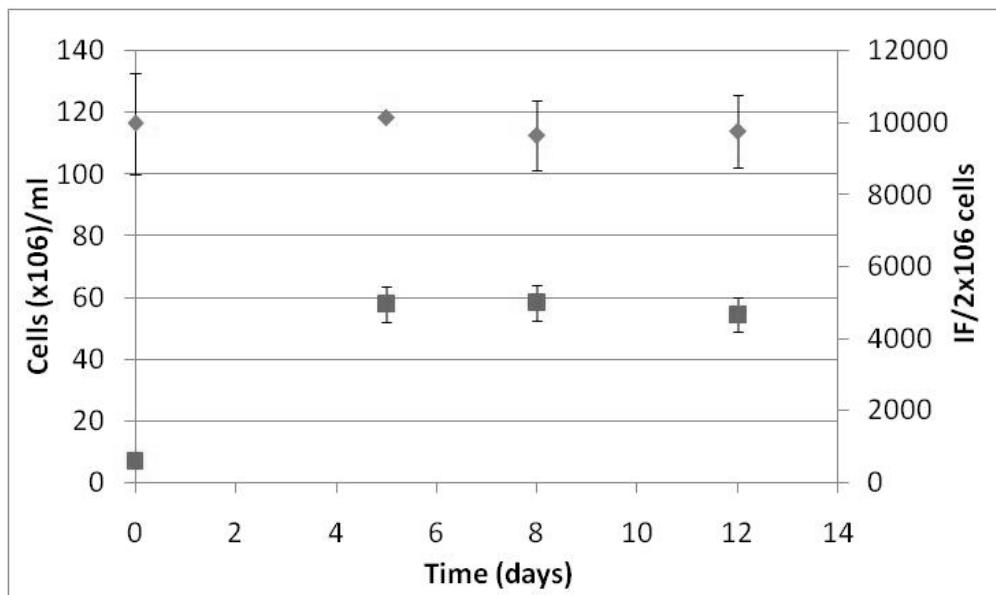


Fig. 7.7 Lipid accumulation induction due to high light intensities stress.

7.5. Final remarks

In this chapter a continuous experiment for production of algal biomass was carried out: the total duration of experiment was 100 days, with a steady state production of biomass of about 60 days, and a productivity of $0.3 \text{ g l}^{-1} \text{ days}^{-1}$. From an experimental perspective, these results indicated that a continuous culture of microalgal biomass is an efficient system to investigate important parameters influencing the cell growth, cell concentration and production performances. In particular, continuous experiments could be carried out under different light intensities or pulsed light regimes, similarly to experiments reported in chapter 6, avoiding problems on growth parameter determination due to self shading of cell concentrations that varies during a batch experiment.

From an applicative perspective, however, during this experiment, under non limited nutrient conditions, the lipid content was low, confirming the hypothesis done in previous chapter, suggesting that for *N. salina* a two step process should be set, if the final product required is the oil for fuel.

In addition, some basic consideration were made to assess the input requirements of a continuous process in terms of energy and nutrients, showing that an integrated approach, by using alternate sources of nutrients, could make feasible the microalgal oil production.

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CHAPTER 8

PROCESS SIMULATION OF MICROALGAL BIOMASS PRODUCTION

This chapter is focused on the simulation of the microalgal biomass production process and photobioreactor using computer models. The models described herein are relatively simple, and they are designed to predict the steady state performance of the photobioreactor in terms of compositions and flow rates in the input and output streams. Process models have been developed using the simulation software Aspen Plus™.

The objectives of this chapter include:

- Setting a non conventional component to represent microalgal biomass
- Developing process models in Aspen Plus™ for microalgal biomass production.
- Using the developed model to study the performance of the photobioreactor by manipulating the process variables
- Changing the geometry of the photobioreactor to determine maximum outputs and productivities

8.1. Introduction

Microalgae are photoautotrophic microorganisms, and their production under controlled conditions requires photobioreactors, which mainly differ from classic bioreactors, such as fermenters or enzymatic reactors, by the need for a light supply in addition to classic chemical growth substrates (Pruvost et al., 2002). The choice of the culture system is affected by several factors: culture medium, water uptake and volume, nutrients, temperature, energy consumption, handling, output products desired. In particular, at the industrial scale, the effective yield has to be achieved. Two of the most relevant problems affecting productivity are the contamination and the presence of competitors. This could be overcome by using closed photobioreactors, that allow a higher system control. On the other hand, as reported in chapter 1, photobioreactors have some unsolved issues, among which the plant cost. Nowadays, the only system potentially feasible, from an economical point of view, seems to be the *raceway pond*. Thus, the solution is to find a simple design for a basic photobioreactor,

coupling higher productivities with a cheaper design. In both cases, light distribution and exposition are crucial to achieve high productivity, as well as the CO₂ supply, in order to obtain an efficient gas transfer in the solution.

In most industries, modeling helps to improve the design and operation of production facilities. In the case of photoautotrophic biotechnology the research is just starting, although various attempts have been made to understand the photosynthetic reaction and its related biomass build-up, in order to obtain higher productivities of living organisms and in conditions different from their natural environment.

A process simulator has the advantage of providing a good insight into the behavior of an actual process and is helpful, in particular, for complex systems with several interacting variables. Process simulation uses mathematical models to generate the description of the state of a system (Raman, 1985). In particular, steady-state simulation by Aspen Plus™ allows to predict the behavior of a process by solving basic mass and energy balances, where reaction kinetics, phase and chemical equilibrium are accounted for. Since a correct computer based mathematical model responds to changes of the same parameters as a real process does, process simulation provides a convenient, inexpensive and safe method of understanding the process without actually experimenting it in a plant. In addition, steady-state simulation is a powerful tool that allows to analyze the effect of changes in process variables. The thermodynamic models and the unit operation models are already built in, but Aspen Plus™ is easily customizable when required (Wooley et al., 1999), in particular concerning the kinetic of the reactions, and the possibility to set non-conventional components.

Looking at an overview of the biodiesel production process, a hypothetical overall system is composed of two main sections (fig.8.1) that can be modelled and investigated with a process simulator: an upstream processing section which is aimed at sequestering CO₂, growing the algae, and producing lipids, and a downstream processing section which includes the pretreatment of the lipids followed by transesterification, separation and finishing to yield biodiesel.

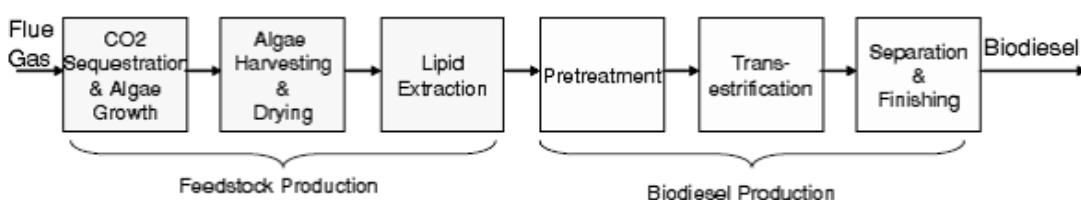


Fig. 8.1. Key elements of the biodiesel production system

Aspen Plus™ applications to microalgae PBRs are lacking: some researchers utilized the software for downstream process simulation, but no information about the growth step are

reported in the literature yet. For instance, Pokoo-Aikins et al. (2010) carried out a process simulation using ASPEN Plus in order to model a two-stage alkali catalyzed transesterification reaction for converting microalgal oil of *Chlorella* species to biodiesel.

The aim of this chapter is to set up the step of biomass production using the process simulator Aspen Plus™: in particular, the biomass component and the microalgal growth kinetic will be modeled.

8.2. Simulation set up

8.2.1. Units

In order to simulate the biomass production process a number of block models was used (Aspentech, 2006):

- Mixer: it combines material streams (or heat streams or work streams) into a single one
- PFR Reactor: RPlug is a rigorous model for plug flow reactors. RPlug assumes that perfect mixing occurs in radial direction and that no mixing occurs in the axial one. RPlug can model one-, two-, or three-phase reactors.
- Split: FSPLIT combines streams of the same type (material, heat, or work ones) and divides the resulting stream into two or more streams of the same type. All outlet streams have the same composition and conditions as the inlet one. The split fraction is specified by the user, for every *substream*.

8.2.2. Process flow diagram

The process flow diagram of the algal biomass production is reported in fig 8.2. The system is composed by a mixer, a reactor, and a split. Two streams of water and nutrients are mixed and fed to the reactor, where algal biomass is produced. The reactor has two output streams, one for the gas and one for the liquid phase. A fraction of the liquid stream, containing the biomass, is recycled back to the reactor as *inoculums* to increase the reaction rate.

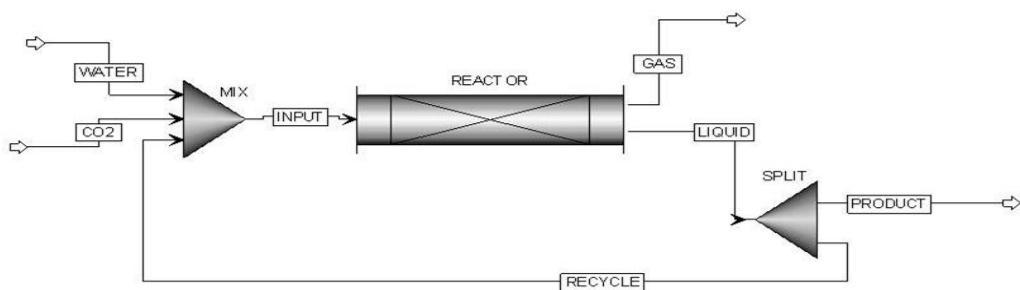


Fig.8.2 Process flow diagram of the microalgal biomass production system.

8.2.3. Component specification

Aspen Plus™ has an extensive database for pure component specification and properties. The database contains parameters of almost 8500 components which include organic, inorganic and salt species (Aspen Tech User Manuals, 2003). The species present in process streams (O_2 , N_2 , H_2O , CO_2) are defined as standard components, except algae that are defined by user. The process simulator allows to set non conventional components, not included in data bank. A non conventional component was set to represent the microalgal biomass, using the specific elemental composition. The algal biomass also contains S and Cl, that were not considered. For *N. salina* the elemental composition is reported in table 8.1.

Table 8.1 Elemental composition of *N. salina* from www.ecn.nl/phyllis

Proximate analysis	[% weight]	Ultimate analysis	[% weight]
Volatile carbon	79.3	Carbon	53.1
Fixed carbon	18.2	Hydrogen	7.3
Ashes	2.5	Nitrogen	8.1
Moisture	0	Oxygen	29.1
		Ashes	2.5

8.2.4. Properties and thermodynamic models

Estimation of accurate thermodynamic properties of pure components and mixtures in a process is crucial for any simulation (Carlson, 1996). Property calculation in Aspen Plus™ can be performed using more than 80 thermodynamic models built in the simulator (Aspen Tech User Manuals, 2003).

Properties for the components in the bioreactor were evaluated using the Non-Random Two Liquid (NRTL) model since the system is a Vapor-Liquid System (VLE) system operating at a pressure lower than 10 atmospheres, with an assumption of the media as a non-electrolyte one (Carlson, 1996). This model was used for the liquid phase, where biomass growth is carried out. Gas solubility was calculated using the Henry's law.

For the non-conventional solid, a model for calculating properties like enthalpy and density must be selected. In our case the HCOALGEN model for enthalpy and the DCOALIGT for density were used, as they are usually applied for processes involving vegetal biomasses. In the HCOALGEN model the Proxanal, Ultanal and Sulfanal characterization of the solid are required. Thus, data from *proximate analysis* and *ultimate analysis* were used for component settings in order to determine the combustion heat, standard heat of formation and enthalpy. The Dulong model for heat combustion was selected, whose results are similar to experimental correlations.

The standard heat of formation was calculated on a heat of combustion basis and specific heat using the Kirov polynomial equation. The standard state was set at 298.15 K.

8.2.5. Reactor design

The photobioreactor was designed as a horizontal flat-plate reactor, as depicted in figure 8.3. Water, nutrients and a fraction of the biomass recycled are the reactor feed. CO₂ is a nutrient for photosynthetic organisms, but is also needed for mixing. The gas is ideally bubbled from the bottom of the reactor, along its length. A pressure control avoids oxygen accumulation in the system. A photobioreactor depth of no more than 20-30 cm was selected.

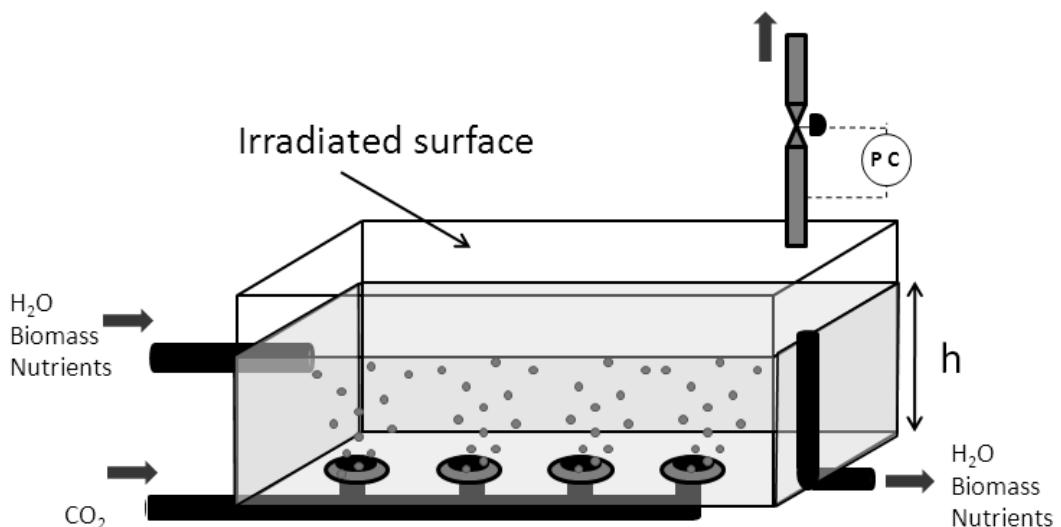


Figure 8.3 Flat plate reactor scheme.

The photobioreactor was simulated as a plug flow reactor (PFR). Perfect radial mixing and perfect axial segregation were assumed. A longer total section of the reactor was set in order to supply a non limiting gas phase. However, the biomass and CO₂ concentrations and the residence time were calculated on the actual liquid volume. Nitrogen required for algal growth was modeled as gaseous nitrogen, only as far as mass balances are concerned. The CO₂ utilized for photosynthesis is that dissolved in the liquid phase (at the saturation concentration).

The state variables of the system, i.e. temperature (25°C), pressure (1 bar) and flow rate, were assigned in the input window, as well as molar compositions of the input streams for the bioreactor are defined in this section.

8.2.6. Design specifications

A given productivity of biomass is imposed as target specification. If this productivity is achieved, the surface needed to absorb all the incident light, can be readily calculated. Thus, with reference to productivity of 1ha, input and output biomass concentrations of about 1

kg/m³ and 1.8 kg/m³ are required. These are reasonable values if compared with typical concentration obtained in lab experiments.

8.2.7. Kinetics

A simplified model for microalgal growth kinetic was assumed. The growth rate is a function of biomass concentration, and nutrients are provided in non limiting concentrations. Inhibition effects due to substrates or products were neglected. The kinetic is thus expressed as:

$$R_i = k \cdot c_i \quad (8.1)$$

where k (d⁻¹) is the maximum specific growth rate and c_i is the microalgal concentration expressed as dry weight (g/L).

In table 8.2 growth rates of microalgal species commonly used for biodiesel purposes are reported.

Table 8.2 Maximum growth rate of several microalgal species.

Microalgalspecies	k [d ⁻¹]	Source
<i>Haematococcuspluvialis</i>	0.64	Garcia-Malea, 2005
<i>Chlorellavulgaris</i>	2.0	Hill, 1981
<i>P. tricornutum</i>	1.51	Molina Grima, 1999
<i>N. oculata</i>	0.68	Chiu, 2009
<i>N. salina</i>	0.72	Boussiba, 1987

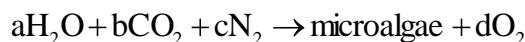
In our simulation, the value of k was retrieved from our experimental data. For *N. salina* species $k=0.5$ d⁻¹ was assumed.

In order to implement the microalgal growth kinetics, a specific Fortran subroutine was written. A thermodynamic routine, which determines the equilibrium CO₂ concentration for each volume, using the Henry equation, was added. The effective gas transfer in the reactor was achieved, thanks to the presence of a bubbling system that ensures a high mass transfer coefficient (see also chapter 3).

8.2.8. Reaction stoichiometry

In order to represent the microalgal growth in the reactor, a stoichiometry of the growth reaction is needed.

The reaction scheme is expressed as:



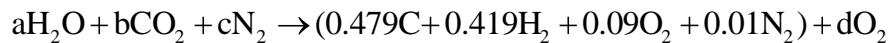
where a, b, c, d are stoichiometric coefficients to be determined.

The microalgal biomass was expressed on the basis of its elemental composition (table 8.2), neglecting sulphur and chlorine content. The molar fractions of carbon, hydrogen, oxygen and nitrogen were used to define the “alga” component.

Table 8.2. *Molar composition of microalgae N. salina, recalculated assuming sulphur and chlorine content as zero.*

	molar %
C	0.479
H ₂	0.419
O ₂	0.090
N ₂	0.010

Thus, the reaction previously reported, can be written as:



The values of a, b, c, d were calculated to balance the reaction stoichiometry:

$$a=0.419;$$

$$b=0.479;$$

$$c=0.01;$$

$$d=0.6$$

and the d value was evaluated by:

$$d=(a+2*b-2*c)/2 \quad (8.2)$$

Consequently, the algae production reaction used in process simulation was:



The software Aspen Plus requires the setting of reaction rates in terms of molar rates. Thus, for conventional components (CO₂, H₂O, N₂, O₂), the production rates (in kg/m³s) were divided by the monomeric unit of algae (9.80 kg/kmol). On the other hand, for non conventional components, mass reaction rates are required. Thus, the production or consumption of each conventional component were calculated by multiplying the molar production rate of biomass by the stoichiometric coefficient of each component.

Thus, the mass production rate of CO₂ is:

$$r_{CO_2} \left(\frac{kmol}{m^3 s} \right) = -0.479 \cdot \frac{R_i}{MW}, \quad (8.3)$$

where R_i is the biomass production rate, MW its molecular weight.

Similarly, for the other components:

$$r_{H_2O} \left(\frac{kmol}{m^3 s} \right) = -0.419 \cdot \frac{R}{MW}, \quad (8.4)$$

$$r_{N_2} \left(\frac{kmol}{m^3 s} \right) = -0.6 \cdot \frac{R}{MW}, \quad (8.5)$$

$$r_{O_2} \left(\frac{kmol}{m^3 s} \right) = 0.01 \cdot \frac{R}{MW}, \quad (8.6)$$

In the Aspen Plus PFR reactor, reaction kinetics reported as kg/m s (for non conventional components) and as kmol/m s (for conventional components) are required; thus the kinetics previously reported were multiplied by the reactor cross sectional area.

8.3. Results

8.3.1. Base Case Simulation

In this section we focus on photobioreactor design, in order to determine the reaction volume required for a given conversion and to calculate the gas flow needed for an adequate mixing.

As reported in section 8.2.6 the input stream entering the reactor has a biomass concentration of 1g/L and the output concentration is 1.8g/L.

Thus, the volumetric flow rate of biomass (P) is given by:

$$\dot{V} = \frac{P}{c_x^{out} - c_x^{in}} = \frac{74.06 \text{kg/h}}{0.8 \text{kg/m}^3} \cong 92.6 \frac{\text{m}^3}{\text{h}} \quad (8.7)$$

The length of the reactor was modified in order to achieve the design specifications on productivity (92.6 kg/h).

A fraction of the biomass produced is recycled, in order to provide a reactor feed concentration of 1 g/L. Thus a design specification was set on the split block, in order to maintain the biomass input concentration wanted (92.6 kg/h requires a water flow rate of 92600 kg/h). An additional design specification was set on the WATER stream in order to regulate the flow rate of water make up added to the recycle stream to the target value corresponds to 92600 kg/h.

At steady state, the biomass entering in the reactor is only derived from the recycle stream, and the total biomass productivity is represented by the output stream after the split.

In table 8.3 the conditions of input streams are summarized.

Table 8.3. Conditions of input streams

	WATER	CO ₂
T [°C]	25	25
P [bar]	1	1
Total flowrate [kg/h]	41785	1000

In table 8.4 the operating conditions of the process are reported.

Table 8.4. Operating conditions and process specifications of the photobioreactor.

Operating conditions	Specifications
T = 25 °C	C ⁱⁿ = 1.0 kg/m ³
P = 1 bar	C ^{out} = 1.8 kg/m ³
	W ⁱⁿ (H ₂ O) = 92600 kg/h

In fig. 8.4 the profile concentration of biomass and CO₂ in the liquid phase along the reactor are reported. The CO₂ concentration decreased from 0.07 g/L to about 0.06 g/L, because of the fixation by microalgae. Note that the lower concentration does not affect significantly the total gas flow rate needed to provide the adequate mixing of the suspension. Results of simulation and the PBR main parameters are presented in table 8.5 and 8.6 respectively.

Table 8.5. Results of simulation

	INPUT	LIQUID	GAS	PRODUCT	RECYCLE
T [°C]	25	25	25	25	25
P [bar]	1	1	1	1	1
Total flowrate [kg/h]	2068	92800	41170	51463	94776
H ₂ O [kg/h]	92600	92279	262.89	41464	50814
CO ₂ [kg/h]	1003	5.61	836.45	2.52	3.09
N ₂ [kg/h]	12000	1.54	11997.16	0.69	0.85
O ₂ [kg/h]	0.02	0.03	145.09	0.01	0.02
ALGA [kg/h]	92.6	168.16	0	75.56	92.6

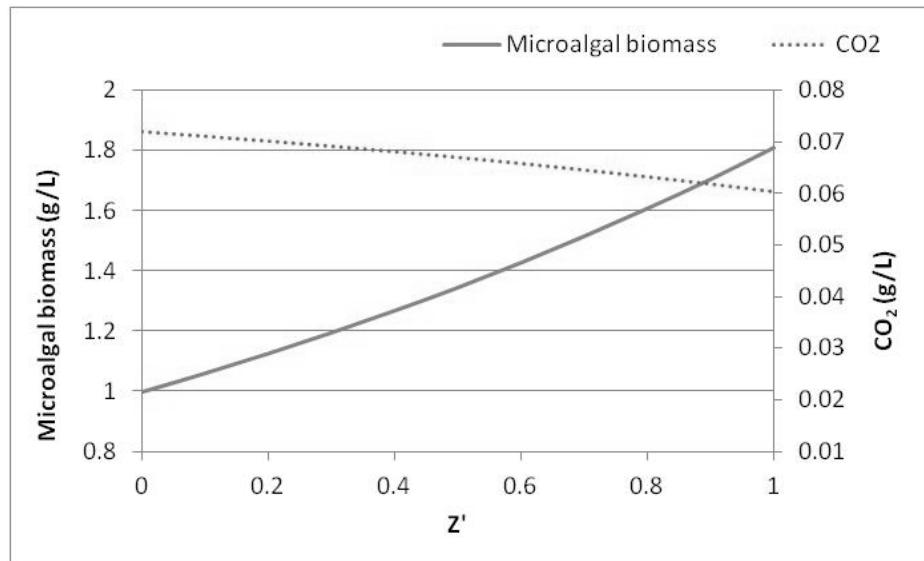


Figure 8.4 CO_2 in liquid phase and biomass concentration profiles in the reactor (the lenght of the reactor is reported as adimensional value $Z'=Z/L$).

Table 8.6 Size of the PFR photobioreactor

$V [\text{m}^3]$	2643
$\tau [\text{h}]$	28.5
$h [\text{m}]$	0.264

As previously assumed, the irradiated surface is 1 ha, so that the depth of the photobioreactor was calculated as 0.264m. If a width of 100m is assumed, the liquid velocity is in the range of few mm/sec, far below the values suggested by the literature (for instance Lehr and Posten, 2009, that reported optimal values in the range of 20–50 cm/s). However, literature data about the flow rates in a photobioreactor are poorly available, due to the lack of working industrial facilities. Anyway, in a photobioreactor with an area of 1 ha, a smaller section has to be designed, by using a serpentine configuration, in order to increase the liquid velocity to acceptable values.

8.3.2. Effect of residence time and input biomass concentration on productivity

In order to study the effect of residence time on biomass productivity, simulations were run by varying the reactor length. Three different value of biomass concentration at the inlet of reactor were investigated ($C_{in} = 0.5 - 1 - 1.5 \text{ g/L}$). Results are displayed in figure 8.5.

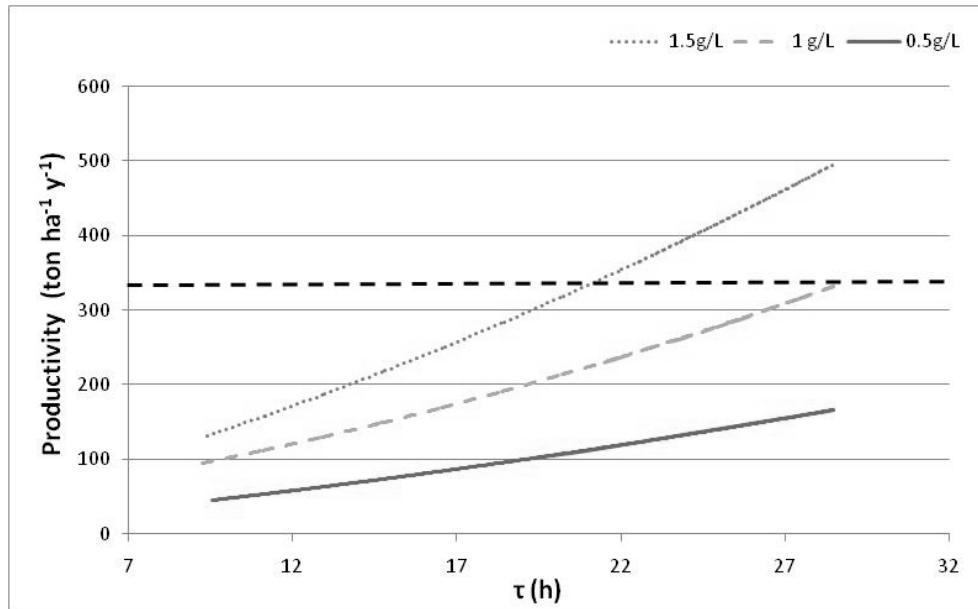


Figura 8.5 Productivity calculated on year basis as function of residence time in the reactor at three different starting concentrations.

As reported in figure 8.5, the productivity (ton ha⁻¹ y⁻¹) increases with the residence time. Concerning the biomass feed to the reactor, higher productivities were obtained with higher biomass concentrations, at constant residence time.

However, productivities higher than 330 ton ha⁻¹ y⁻¹ are above the maximum thermodynamic limit which has been, calculated on the sunlight irradiance on the Earth surface. Thus, they can't be reached in practice.

In addition, the simulation results allow to calculate the depth of the reactor needed to absorb the light available, which is a function of the surface exposed of the reactor liquid volume (1 ha, as hypothesized previously).

Results are shown in figure 8.6.

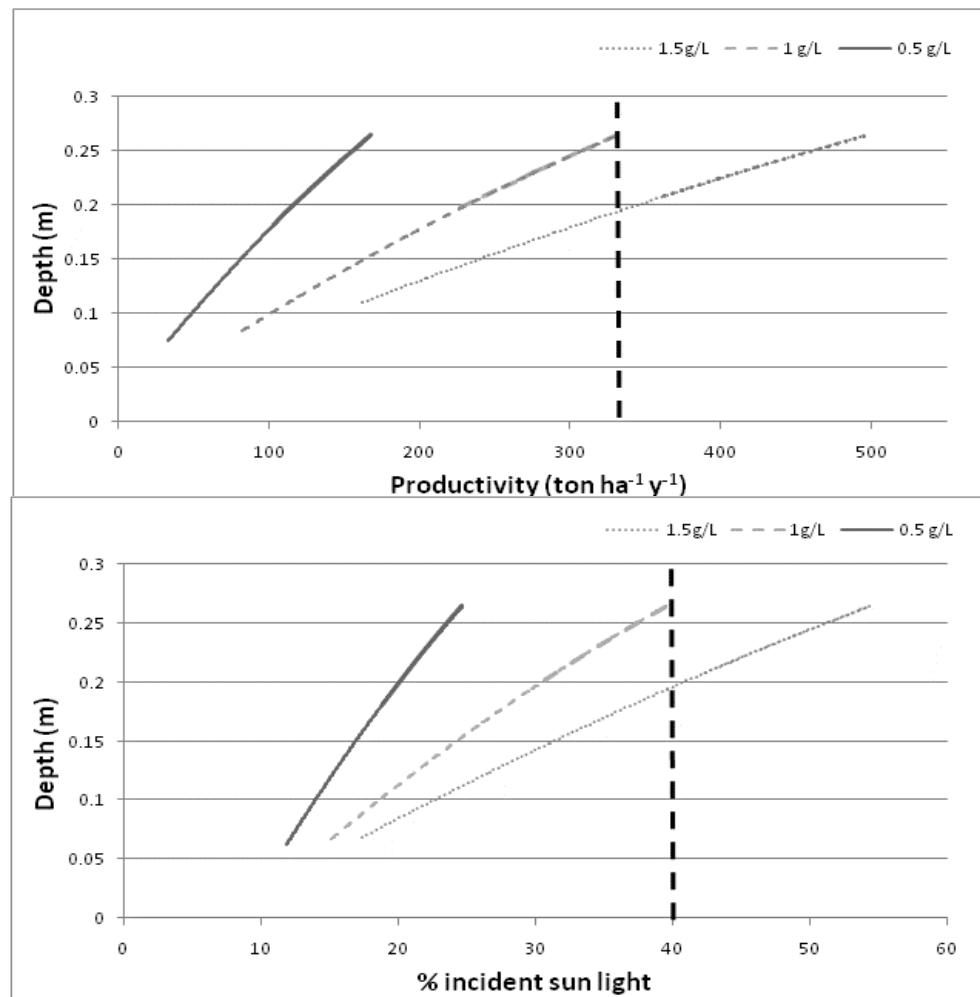


Figure 8.6 Reactor depth needed to achieve a specific productivity (8A) and to absorb the available light (8B), calculated on three different biomass concentrations.

The photobioreactor depth is strongly affected by the input biomass concentration, as reported in figure 8A: by considering a constant photobioreactor surface, the depth decreases with concentration. Note that 25 cm can be regarded as an upper limit of this depth. Above this value the light uptake becomes unefficient, owing to a loss of light due to the optical density of the suspension. Thus, we can conclude that at input concentration of about 0.5 g/L, the culture cannot reach a concentration near to the theoretical limit with reasonable residence time and reactor lengths. On the opposite, at higher concentrations, a depth higher than 20 cm is useless to increase the productivity because the thermodynamic limit imposed by the irradiance of sunlight of the earth surface (330 ton/ha⁻¹ y⁻¹) is readily achieved.

8.4. Remarks on energy content of microalgae and energy recovery

In view of biofuel production, we need to consider that the lipid productivity is a fraction of the biomass productivity previously calculated, and downstream processes are required. The simulation of downstream processes is, however, far beyond the scope of the present chapter. However, some remarks on energy recovery can be done considering the energy content of the microalgal biomass. The energetic feasibility of the microalgal biomass production is the unsolved issue, and a number of researchers are working on energy recovery, in particular on the downstream energy demand, that seems to be the bottleneck of the feasibility of the process (see also chapter 1). The step with a higher energy consumption is the dewatering, and LCA studies showed that the process could be sustainable only by avoiding this step. However, some preliminary considerations could be done considering the biomass production step: after the extraction of lipids, the energy content of the residual biomass could be used to support the energy demand of the process.

Average literature data of the energy content of algal biomass and algal oil are 20 MJ/kg (Lehr, 2004) and 37.05 MJ/kg respectively as reported in table 8.7.

Table 8.7: energy content of oil from microalgae

Oil energy content (MJ/kg)	Source
37.20	accesstoenergy.com/view/atearchive/s76a5002.htm
38.00	www.houstontech.org/en/releases/printview.asp?372
37.00	www.biomatnet.org/publications/1996repa.pdf
37.05	AVERAGE DATA

The models of the process simulator, used for non conventional components, usually calculate the formation enthalpy, the specific heat and density of biomass. However, although these models are specifically formulated for the biomass, they are usually modeled on coal and, consequently, the simulation output resulted incorrect for microalgal biomass. Thus, for *N. salina* biomass, the enthalpy formation value and specific enthalpy have to be set by the user, based on literature data, in order to calculate the heating value of the residual biomass after lipid extraction.

The low heating value of the residual biomass was calculated as:

$$LHV_{bio} = \frac{LHV_{alg} - xLHV_{oil}}{1-x} \quad (8.8)$$

where LHV_{alg} and LHV_{bio} are the energy content (combustion heat) of biomass before and after the extraction respectively, LHV_{oil} is the heating value of the oil and x is the fraction of oil in the biomass.

Results of these preliminary calculations for *N. salina* are reported in table 8.8.

Table 8.8. Theoretical microalgal oil productivity

Max biomass productivity (ton ha ⁻¹ y ⁻¹)	LHV _{alg} (MJ kg ⁻¹)	LHV _{oil} (MJ kg ⁻¹)	Oil fraction	Oil productivity (ton ha ⁻¹ y ⁻¹)	LHV _{bio} (MJ kg ⁻¹)
330	20	37	0.5	165	3.00
			0.4	132	8.67
			0.3	99	12.71

8.5. Final remarks

Process simulation allowed to predict the behaviour of the photobioreactor by using basic mass and energy balances, reaction kinetics, phase and chemical equilibrium. The microalgal production process is not fully understood, and actual plants are not working yet. Thus, not only a better comprehension of the biology of these organisms is required, but also some evaluations of the feasibility of the process at the industrial scale could be helpful. To this aim, a basic process of microalgal biomass production in a photobioreactor was set up and simulated with the most commonly used simulation software, Aspen Plus. In order to do that simulate a photobioreactor with the process simulator, a non conventional component was set (microalgal biomass), and a *Fortran* subroutine was written in order to simulate the kinetic and stoichiometry of growth reaction in the reactor. Some indications about the reactor geometry, in particular the depth of the reactor, was obtained by changing the length and the residence time of the photobioreactor.

However, it is important to consider the thermodynamic constraint imposed by solar light irradiation on the exposure surface of the reactor.

Sensitivity analysis showed that, at the same productivity, an higher inlet concentration of biomass requires smaller reacting volume and residence time.

The simulation of biomass production step, anyway, is strictly related to the species considered, not only depending on the reactor geometry. In fact, the species used in this simulation is *N. salina*, that accumulates lipids only under stressed conditions. Consequently, for this species, an additional block after biomass production step is required, under different conditions of nutrients or light, in order to increase the lipid concentration in the biomass.

The simulation of the biomass production step, however, was satisfactory, and could be applied for several algal species, only considering and modifying the specific elemental composition and the growth rate.

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CONCLUSIONS

Fuels from algae certainly have a great potential and can be viewed and developed to become competitive with respect to petroleum-derived fuels. However, although microalgae offer a number of advantages, a sustainable algal biofuel industry is at least one or two decades away from maturity, as no commercial scale units are currently in operation. Thus, to effectively address process bottlenecks associated with microalgal biodiesel production, a leap in both fundamental knowledge and technological applicability is required.

This PhD thesis was focused on increasing the scientific knowledge in view of microalgal massive production, as far as the species selection and the design of photobioreactors are concerned.

Accordingly, experimental activities were carried out to set up and optimize materials and methods, with particular attention to the techniques for lipid determination. A semibatch experimental apparatus to ensure a non limiting CO₂ supply was first designed and built. Several wild type species were screened, both from salt water and fresh water environments. Among others, *Nannochloropsis salina*, a marine one, was selected for its biomass concentration, growth kinetic parameters and lipid content. This species was utilized as a model to understand phenomena and key operating variables involved in biomass growth and lipid formation, which are essential in the development of large-scale photobioreactors for natural oil massive production from microalgae. The growth media conditions were deeply investigated for this species and, accounting for both thermodynamic and mass transfer considerations, it was shown that carbon dioxide is one of the main nutrients affecting growth, as well as nitrogen, which is also involved in lipid accumulation. In fact, the lipid concentration in *N. salina* was maximized by applying nitrogen deprivation.

Even if the media conditions play a crucial role in biomass and lipid production, from a large scale perspective the supply of basic nutrients should be considered as well, in order to make the process economically viable and sustainable. Accordingly, the utilization of industrial byproducts or wastes to feed algae photobioreacotrscould potentially provide cost-effective and sustainable means of algal growth for biofuels, also including the potential of simultaneous bioremediation. An interesting scenario, in this respect, is to exploit flue gases from combustions and nutrients present in industrial byproducts or wastewaters: both of them could be fed to a photobioreactor where microalgae growth is sustained. To this aim, we

selected a freshwater species able to grow fast and accumulate lipids, and we verified the capability of that species to exploit CO₂ from combustion gases and nitrogen from industrial process waters.

During this research project, the feasibility of the mixotrophic cultivation was also investigated for two species: *N. salina* and *C. protothecoides*, a freshwater one. Both of them were found able to grow in mixotrophic cultures, and to use organic substrates alternative to sugar, such as ethanol, acetate and glycerol. We focused in particular on glycerol since this is a byproduct of biodiesel production and could be profitably recycled in an industrial algal biomass production process. However, microalgae cultures need to be supplied with high CO₂ concentrations to maximize their photosynthetic productivity: in this thesis we showed that the presence of both high CO₂ concentration and organic substrate inhibit mixotrophy, likely because of an inhibition of respiration. Experimental runs were carried out also under dark/light cycles, to mimic the real situation. Thus, the ideal compromise for an outdoor photobioreactor working with a night-day cycle to reach the maximal biomass productivity will be to have an autotrophic growth with excess CO₂ during the day and a heterotrophic growth in glycerol without adding CO₂ overnight. In addition, we observed that the supply of glycerol lead to an increased lipid content in algal biomass, without the need of nitrogen deprivation.

As for nutrients, also light has a major influence on algae cultivation: if too low, it limits algae growth while, if in excess, it drives to oxidative stress. Light use efficiency in large scale units must thus be optimized to achieve a sufficient productivity to make the system economically sustainable. The influence of different light intensities on growth and lipids productivity of *N. salina* was investigated using a flatbed photobioreactor designed to minimize cells self-shading. Furthermore, the alternation of light and dark cycles with different frequencies in order to mimic illumination variations due to mixing was studied. Results showed that, if light is provided with appropriate frequency, even a very strong light, far beyond the saturation point of photosynthesis, can be exploited to get efficient growth. From an industrial perspective, these results suggested that an appropriate design of the photobioreactor, aimed to ensure a suitable mixing, could enhance the photosynthetic efficiency, and, consequently, the overall biomass productivity.

All the experimental results obtained in this thesis allowed a better comprehension of the biology of microalgae; anyway, from an industrial perspective, some considerations of the feasibility of the process at large scale could be helpful. To this aim, a continuous reactor is required, because the effectiveness of continuous cultures is greater than for batch ones. From this perspective, a continuous experiment for algal biomass production was carried out: the total duration of the run was 100 days, with a steady state production of biomass of about 60 days, and a productivity of 0.3 g l⁻¹ days⁻¹ of dry weight. These results indicated that a continuous culture is an efficient system which not only demonstrates the feasibility of

microalgal continuous production, but also can be used to investigate important parameters influencing the cell growth, cell concentration and production performances. However, during this experiment, under non-limited nutrient conditions the lipid content was low suggesting that, in the case of *N. salina*, a two step process should be set, if the final product required is the oil for fuel.

In addition, some basic consideration were made to assess the input requirements of a continuous process in terms of energy and nutrients, showing that an integrated approach, by using alternate sources of nutrients, could make the microalgal oil production feasible.

During this work, also a process model was developed. Process simulation allowed to predict the behaviour of the photobioreactor by using basic mass and energy balances, reaction kinetics, phase and chemical equilibrium. Indications about the reactor geometry, in particular the depth of the reactor, were obtained to maximize the productivity. In addition, the model allowed to perform sensitivity analysis, showing that, for a given productivity, a higher inlet concentration of biomass requires a smaller reacting volume and residence time. The model developed can be applied for several algal species, only considering and modifying the specific elemental composition and the specific growth rate of the species of interest.

In summary, the results obtained in this PhD thesis may be useful to design a large-scale unit, although more research is still needed before the potential offered by microalgae as source of biodiesel can be exploited. The energy duty, supply and light absorption of the process must be considered first. It was shown that the limit of biomass yield per unit land used, which depends on light availability rather than on the intrinsic growth rate, may impact on the reactor geometry, which should be kept as simple as possible also for economic reasons. Furthermore, the oil content of the species is crucial for an effective recovery of biomass in the downstream process. Also the supply of nutrients is a key issue and wastewaters should be used whenever possible.

In a long-term vision, production chains with net energy output have to be identified and continuous research and development are needed to reduce the cost in all steps of this production process. Major breakthroughs are still lacking in view of the design and development of technologies that can reduce costs while increasing yields. Only well-funded, coherent and long-run research programs will eventually attain this goal: in the longer term, also genetic engineering is likely to have the greatest impact on the feasibility of algal biofuels.