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Ciclo XVI

Microalgae biomass production at different growth conditions assessing the lipid content and fatty acid profile for feed, food and energy applications

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Summary

Abstract

L'utilizzo delle microalghe come risorsa di cibo, mangimistica e fonte di energia rinnovabile, ha ricevuto un considerevole interesse negli ultimi anni. Tuttavia, è necessario ottimizzare le condizioni di crescita delle microalghe per ottenere una produttività sostenibile e fattibile dal punto di vista economico. Nel seguente lavoro di tesi, è stata focalizzata l'attenzione sul miglioramento della produttività lipidica di due specie di microalghe *Botryococcus braunii* e *Phaodactylum tricornutum*, ed è stato valutato il profili lipidico del ciano batterio *A. maxima* comunemente conosciuto come Spirulina, coltivato alle latitudini del Mediterraneo. Questo lavoro è stato suddiviso in quattro capitoli: 1) Crescita di due ceppi di *Botryococcus braunii* in reflui domestici prima e dopo il trattamento; 2) Effetto delle differenti variabili ambientali sulla quantità e sulla qualità dei lipidi di *B. braunii* coltivato su piccola e grande scala; 3) Strategie per migliorare il contenuto e il profilo lipidico di *Phaeodactylum tricornutum*; 4) Contenuto lipidico e profilo degli acidi grassi di *A. maxima* coltivata nel sud Italia.I due ceppi di *B. braunii* hanno mostrato un buon rendimento di crescita in acque reflue, e il più elevato contenuto lipidico (24±2,4 %) è stato registrato dal ceppo UTEX LB 572 cresciuto nel refluo influente. Sia i ceppi UTEX che IBL hanno dimostrato un equilibrio tra la quantità di acidi grassi saturi e insaturi adatti come fonte di carburante biologico.*B. braunii* ha mostrato un contenuto lipidico della biomassa significativamente più elevato usando starvazione di azoto con un valore massimo del 65±4.3%. per quanto riguarda la produttività lipidica, la starvazione di azoto non ha migliorato la produttività lipidica rispetto a una coltura con il 3% di insufflazione di CO_2. Anche l'utilizzo di $NaHCO_3$ come fonte di C, in sostituzione della CO_2 non ha mostrato risultati statisticamente significativi in termini della produttività lipidica di Bb. L'aggiunta di $NaHCO_3$ ha migliorato il contenuto dell'acido γ-linolenico con valori del 37±4% rispetto agli acidi grassi totali. L'acido γ-linolenico è importante per le sue proprietà anti-infiammatorie e immuno-modulatorie, e può avere molte applicazioni nel campo alimentare. Il contenuto e la produttività lipidica di *P. tricornutum* è significativamente più elevato utilizzando un terreno di crescita con starvazione di azoto. La produzione in vasche sotto serra ha mostrato i più alti valori in contenuto di EPA(25,7%) rispetto ai TFA e bassi costi di produzione con una alta produttività della biomassa.*A. maxima* è stata coltivata per due anni (2012-2013) a partire da Giugno fino a Novembre, e ogni mese è stato valutato il contenuto e il profilo lipidico della biomassa prodotta. Il contenuto lipidico medio tra le due stagioni produttive è stato, rispettivamente, di 8.0±3.2% (dw) nel 2012 e 8.3± 2.2%(dw) nel 2013, con i valori più alti registrati a settembre di entrambi gli anni. Il profilo lipidico non ha mostrato delle differenze statisticamente significative tra gli anni, e la somma dell'acido linoleico e γ–linolenico è stata del 50% del TFA(acidi grassi totali). *B. braunii* e *P. tricornutum*, coltivate nel sud Italia, con tecnologie a basso costo, ha mostrato una produttività lipidica di 4,4 e 7,0 (ton/ha/y rispettivamente, valori considerevolmente molto più alti rispetto alla produttività lipidica dell'olio di semi di girasole, dell'olio di oliva, dell'olio di soia e dell'olio di mais. *A. maxima* ha mostrato una produttività lipidica di 0,96 (ton/ha/y) simile a quella dell'olio di semi di girasole (0,77ton/ha/y). *A.maxima, P.tricornutum e B.braunii* sono una fonte promettente di olio che potrebbe essere utilizzato come ingrediente funzionale nell'industria alimentare o nel mercato come supplemento per il cibo. Queste microalghe potrebbero essere coltivate alle nostre latitudini un costo di produzione relativamente basso e con buone caratteristiche nutrizionali

Abstract

The potential of microalgae as a source of food, feedstock and renewable energy has received considerable interest, but if microalgae production is to be economically viable and sustainable, further optimization of mass culture conditions are needed. In this work the attention was focused on the improvement of lipid productivity of two microalgae species *Botryococcus braunii* and *Phaeodactylum tricornutum*, and evaluation of lipid productivity and fatty acid profile of the cyanobacterium *Arthrospira maxima*, formely known as Spirulina, cultivated at the mediterranean latitudes. The thesis work consists of four experiments: 1) Growth of two strains of *Botryococcus braunii* (UTEX LB 572 and IB 115) in domestic effluents before and after pretreatment stage; 2) Effects of different environmental variables on quantity and quality of lipids of *B. braunii* cultivated in small and large scale; 3) Strategies to improve lipid content and fatty acid profile in *Phaeodactylum tricornutum*; 4) Lipid content and fatty acid profile of *A. maxima* cultivated in south of Italy.

The two strains of *B. braunii* showedgood growth performances into the waste effluents with the highest value of lipid (24%) evidenced by UTEX LB 572 strain grown in influent medium. Both strains have showed a balance between saturated and unsaturated fatty acids suitable as a source of biofuel.

B. braunii was shown to be capable of accumulating lipids as 65% of the biomass using nitrogen starvation, while supplying additional CO2 (3%) in the culture medium with nitrogen starvation did not improved lipid productivity. NaHCO3 as a source of C, instead of CO2, improved the γ-linoleic acid content of *B.braunii* (37±4% on total FA). γ-linolenic acid is important for its anti-inflammatory and immune-modulating activity and can have applications in food and feed.

The lipid content and lipid productivity of *P. tricornutum* was significantly enhanced using nitrogen starvation. Production in ponds under greenhouse showed the highest value in EPA content (25,7%) on total FA and low production costs with high biomass productivity.

The obtained results suggested that nitrogen limited algal cultivation is feasible and could be a useful strategy for producing lipid as biofuel feedstock or fatty acids for food and feed.

A. maxima was grown for two years (2012-2013), from June to November, monthly evaluating lipid content and lipid profile of the obtained biomass. The mean value of lipid content in the two seasons were 8.0±3.2 and 8.3 ± 2.2 %(dw), in 2012 and 2013, respectively. The highest values was in September of both years. The lipid profile did not shown significant differences through the year and the sum of linoleic and γ–linolenic acids was about 50% of total FA.

B. braunii and *P. tricornutum* cultivated, with low-cost technologies showed lipid productivityconsiderably more higher than Sunflower, Olive, Soy and Corn. *A. maxima* showed a lipid productivity of 0.96 t/ha/y, value close to sunflower lipid productivity 0.77t/ha/y.

Thereforemicroalgae *A.maxima, P.tricornutum e B.braunii* are promising source of oil to be used as a functional ingredient in food industry or for the market of food supplements. These microalgae could be cultivated at our latitudes at relatively low cost of production with good nutritional characteristic

Chapter 1: Introduction

1.1 Introduction

In a bio-based economy, agricultural crops are not only used for production of food and feed but also for chemicals, materials and biofuels. Owing to the scarcity of available fossil feedstocks for non-food products, there is an increasing demand for supply of bio-basedfeedstocks for both food and non-food ingredients. As a result, there is a debate on whether production capacity of biomass for both food and non-food products can be sufficient.

Especially with the world population rising to 9 Billion in 2050, with many inspiring to a western life style and diet, this challenge should be addressed urgently(Draismaa *et al.,* 2013). Availability of arable land, fresh water and fertilizer usage and effects on biodiversity are major factors that need to be taken into account in sustainable agriculture (Godfray *et al*., 2010; Tilman *et a*l., 2011).

Microalgae are considered one of the most promising feedstocks for sustainable supply of commodities for bothfood and non-food products (Wijffels and Barbosa, 2010; Williams and Laurens, 2010; Tredici, 2010; Milledge, 2011; Hoekman *et al*., 2012). Microalgae do not need to be grown on arable land, can be grown on seawater and on residual nutrients, have a high areal productivity and are also rich in oils, proteins and carbohydrates; moreover the algal biomass can be fractionated into both food and non-food products via biorefinery (Wijffels *et al*., 2010).

Eukaryotic microalgae and photosynthetic cyanobacteria have a great potential for productionof food commodities such as edible oils, proteinand starch. Only eukaryotic microalgae are capable ofnatural triacylglyceride production, unlike the prokaryoticmicroalgae (cyanobacteria) and macroalgae.

Oleaginous eukaryotic microalgae can accumulate up to50–70% oil (Chisti, 2007; Hu *et al*., 2008; Bondioli *et al*., 2012).

1.2 Microalgal oils

For most applications in food products, oil present astriacylglycerides (TAG) is preferred, which is the lipidclass used by oleaginous eukaryotic micro-organisms tostore their fatty acids under stress conditions(Ratledge, 2004). Theaccumulation of fatty acids by oleaginous photoautotrophicmicroalgae is well established and recentlyreviewed (Hu *et* al., 2008; Griffiths and Harrison, 2009; Rodolfi *et a*l. 2009). Under stress conditions; such aslimitation or depletion of specific nutrients, a sub optimalpH, high salinity and high light conditions, a reduction ofthe degree of unsaturation of intracellular fatty acids wasshown (Stephenson*et al*., 2010; Breuer *et al*., 2012; Santos et al., 2012; Lamers *et al.,* 2010; Pal *et a*l., 2001). Accumulation of fatty acids inTAG under nitrogen limitation or depletion has beenconfirmed for a number of microalgae (Bondioli *et a*l., 2012,Pruvost *et al*. 2011;Stephenson et al., 2010,Breuer et al., 2012,Yu et al., 2009).

The microalgal fatty acids listed in Table 1, provided theyare present in TAG, offer options to partly replace functionsof the currently used vegetable oils. For instance thepresence of linoleic (C18:2) and alpha-linolenic acid(C18:3) may partly substitute the essential fatty acidcontribution from rape seed (canola), soy or sunfloweroils, while palmitic acid (C16:0) in microalgal oils cancontribute to structuring in food products. The presenceof long chain polyunsaturated fatty acids (LC-PUFA)such as eicosapentaenoic acid (C20:5), docosahexanoic acid (C22:6) and the C20:5 precursor, stearidonic acid (C18:4) are of interest because of their cardiovascularhealth benefits (Mozaffarian and Rimm, 2006; Harris *et al*., 2008).

Table 1. Comparison of the fatty acid (FA) profiles of various oleaginous microalgae and major vegetable oils. Specific fatty acids present below 1% were not included. All microalgae were cultivated under nitrogen deplete or limiting conditions, which resulted in a total FA content above 20% (w/w) in all cases, suggesting the presence of TAG. FA profiles from different strains of microalgae species were pooled, with the exception of Neochlorisoleabundans, where results from the same strain were used. In cases where results were pooled, FA content was averaged and relative standard deviation was calculated (without subscript indicates a relative standard deviation in 1–30% range).(Rene *et al.* 2013)

Fatty acids	C10:0	C14:0	C16:0	C16:1	C16:2	C16:3	C18:0	C18:1	C18:2	C18:3	C18:4	C20:3	C20:4	C20:5	C22:6	Ref.
Microalgae																
Ankistrodesmusfalcatus			11	1	3		3	53	14	12						Griffiths *et al*. 2012
Chlorellavulgaris			17	1c	3	6	4c	48	9	13						Breuer *et al.*, 2012, Griffiths *et al*. 2012
Chlorellazofingiensis			15	1	4	2	3	47	17	8						Breuer *et al.*, 2012
Scenedesmussp.			19c	3d	4	2d	5	48	12d	4d	1					Breuer *et al.*, 2012, Griffiths *et al*. 2012
Neochlorisoleoabundans			23	2d	2c	2	4	44	21	4a						Breuer *et al.*, 2012, Griffiths *et al*. 2012
Cylindrothecafusiformis		6	35	35		1		9	3			2	5	3		Griffiths *et al*. 2012
Isochrysissp		20d	16	3			2d	33	3	4	10			2	13a	Breuer *et al.*, 2012, Griffiths *et al*. 2012
Nannochloropsissp.		5	40	29			1	16	1				2	4		13, Griffiths *et al*. 2012
Phaeodactylum tricornutum	5b	5c	24	47	2c	2	1c	5d	1c				1	9		Breuer *et al.*, 2012, Griffiths *et a*l. 2012 Zendejas *et al*., 2012
Thalassiosirapseudonana	7b	10	28	32	4			4			4			9	1	Zendejas et al., 2012
Rapeseed (canola)			4				2	62	22	10						Knothe *et al*., 2010
Palm			44				4	39	11							Knothe *et al*., 2010
Soy			8				4	24	53	6						Knothe *et al*., 2010
Sunflower			6				4	25	63							Knothe *et al*., 2010

a Solely present in FA profiles (Breuer *et al*., 2012).
b Solely present in FA profiles (Zendejas *et al*., 2012).
c Relative standard deviation 30–40%.
d Rel. St. Dev. 40–106%.
e FA profile of neutral lipids was used (Bondioli *et al*., 2012)

Fish and fish oil are the common sources of long-chain PUFAs but safety issues have been raised because of the possible accumulation of toxins in fish (Apt. and Behrens, 1999). Moreover, the application of fish oil as food additive is limited due to problems associated with its typical fishy smell, unpleasant taste and poor oxidative stability (Certik and Shimizu, 1999; Luiten *et al*., 2003; Abril *et al*., 2003).

For some applications, fish oil is often suitable because of the presence of mixed fatty acids (Robles *et al*., 1998). As PUFAs are found in fish originating from microalgae consumed in oceanic environments, it is logical to consider microalgae as potential sources of PUFAs (Jiang *et al*., 1999).

Table 2presents the microalgal PUFAs of particular interest: however, currently, DHA is the only algal PUFA commercially available. Indeed, even if species have demonstrated industrial production potential of EPA (*Porphyridiumpurpureum, Phaeodactylumtricornutum, Isochrysisgalbana, Nannochloropsis sp. and Nitzschialaevis*) (Robles *et al.,* 1998; Molina *et al*., 2003; Wen and Chen 2003), no purified algal oil is currently economically competitive with other sources (Apt and Behrens, 1999; Zittelli *et al.* 1999; Belarbi *et al*., 2000).The same problem exist with γ-linolenic acid (GLA) and arachidonic acid AA.

DHA is an ω3 fatty acid found in different tissues of the body. It is a major structural fatty acid in the grey matter ofthe brain and in the retina of the eye, and is a key componentof the heart tissue. DHA is important for correct brainand eye development in infants and has been shown to supportcardiovascular health in adults (Kroes *et al*., 2003;Ward and Singh, 2005). It is found infew food such fatty fish and organicmeat; it also occurs naturally in breast milk but is absentin cow's milk. Since 1990 onwards, a number of health and nutrition organizations specifically recommended the inclusionof DHA in infant formula for preterm and fullterminfants. The world wholesale market for infant formula isnow estimated to be about US$ 10 billion per annum (Ward and Singh, 2005).

Martek's DHA oil for this application (DHASCO; Martek,Columbia, MD, USA) comes from *Crypthecodiniumcohnii*and contains 40–50% DHA but no EPA or other long-chainPUFAs (Jiang et al., 1999, Ward and Singh, 2005, Ratledge, 2004). The heterotrophic process uses anumber of fermenters, each about 100 m^3, and meets strictmanufacturing conditions that follow the US Food and DrugAdministration's (FDA) current Good Manufacturing Practice(cGMP) regulations. The production for 2003 was 240 t (Ratledge, 2004)and formulas containing Martek's oil are available in morethan 60 countries worldwide (e.g., United Kingdom, Mexico,China, United States and most recently, Canada).Moreover, OmegaTech (USA), also owned by Martek,exploits *Schizochytrium* to produce a low-cost oil formerlyknown as DHA Gold (10 t in 2003; Ratledge, 2004). The oil is currentlyused as an adult dietary supplement in food and beverages,health foods, animal feeds and maricultural products. Example of these foods are cheeses, yogurts, spreads and dressings,and breakfast cereals. Other markets include foods for pregnant and nursing women and applications in cardiovascularhealth (Ward and Singh, 2005).

Finally, the Nutrinova process (Frankfurt, Germany) uses*Ulkenia sp*. which grows in 80-m3 fermenters. The oil issold under the name of DHActive (Ratledge, 2004; Pulz, Abstr. Eur.Workshop MicroalgalBiotechnol., Germany, p. 35, 2005).

Contribution to product taste by microalgal oils and any adverse effect of less common fatty acids in the fatty acid profiles will need to be evaluated before application (Spolaore *et al*., 2006).

Table 2. Particularly interesting microalgal PUFAs. The most interesting PUFA in microalgae

PUFA	Structure	Potential application	Microorganism producer
γ-Linolenic acid (GLA)	18:3 ω6, 9, 12	Infant formulas for full-term infants Nutritionalsupplements	Arthrospira
Arachidonic acid (AA)	20:4 ω6, 9, 12 ,15	Infant formulas for full-term/preterm infants Nutritional supplements	Porphyridium
Eicosapentaenoic acid (EPA)	20: 5 ω3, 6, 9, 12, 15	Nutritionalsupplements Aquaculture	Nannochloropsis, Phaeodactylum, Nitzschia
Docosahexaenoic acid (DHA)	22: 6 ω3, 6, 9, 12, 15, 18	Infant formulas for full-term/preterm infants Aquaculture Nutritional supplements	Crypthecodinium, Schizochytrium

1.3 Microalgae biology

Microalgae are a huge group of photosynthetic microorganisms from freshwater, brackish and marine systems, typically unicellular and eukaryotic. Some of the most significant groups of algae are green algae (*Chlorophyceae*), red algae (*Rhodophyceae*), diatoms (*Bacillariophyceae*), and brown algae (*Phaeophyceae*). Although cyanobacteria (blue green algae) are classified to the domain of Bacteria, being photosynthetic prokaryotes, often they are considered as "microalgae"(Medlin *et al*., 2007).

Eukaryotic microalgae can be either autotrophic or heterotrophic. Autotrophic microalgae require only inorganic compounds such as CO_2, N, S, P, and light as an energy source for their growth and development. They convert captured solar energy into biomass (photosynthesis) with an efficiency that generally exceed those of terrestrial plants (about 3 % reported for marine microalgae against 0.2–2 % for terrestrial plants)(Stephenson *et al*., 2011). Some photosynthetic microalgae are mixotrophic, meaning they are able simultaneously to perform photosynthesis and to catabolize exogenous organic nutrients, but some species are not truly mixotrophs, because they but have the ability of switching between phototrophic and heterotrophic metabolisms in relation to the depending on environmental conditions(Kaplan et al., 1986; Perez-Garcia *et al*., 2011).

With these simple growth requirements, microalgae can sustainably generate lipids, proteins and carbohydrates at a large scale, offering promising environmentally friendly alternatives to the current consumer products.

Microalgae active compounds, such as carotenoids, phycobilins, fatty acids, polysaccharides, vitamins and peptides, can be used in feed, food, nutraceutical, cosmetics and pharmaceutical industries (Gouveia *et al*., 2006).

The chemical composition of microalgae showed to be greatly variable also in agreement with some environmental factors, such as water temperature, salinity, light, nutrients availability and also to the production technologies. In outdoor cultivation most of the environmental parameters vary according to the season stimulating or inhibiting the biosynthesis of several nutrients; while in close photobioreactor systems the cultivation occur in well controlled conditions, but it is usually more expensive (Marinho-Soriano *et al*., 2006; Carlucci *et al*.,1999).

1.4 Microalgae cultivation for food production

Commercial large-scale production of microalgae started in the early 1960s in Japan with the culture of *Chlorella* used as a food additive, followed by the cyanobacterium*Arthrospira*. Only after 1980 large-scale algae production facilities were established in Asia, India, USA, Israel and Australia (F.A.O. 2008). Commercial microalgae farms for value-added products are usually conducted in open ponds under autotrophic conditions in location having all the year relatively warm temperature or in fermenters under heterotrophic conditions.

Microalgae showed some important advantages respect to conventional land plants: they have much higher biomass productivities (around 10–50 times higher) and CO_2 fixation rate, moreover arid or low quality agricultural land can be used for their cultivation (Singh and Gu, 2010; Scott *et al*., 2010).Although microalgae cultivation is carried out in aquatic environment, they use less water than terrestrial crops, so the freshwater consumption is strongly reduced. Furthermore, microalgae may be cultivated in brackish and sea water avoiding herbicide or pesticide application, and reducing the needs of external nutrients (NH_4, NO_3 and P) (Aslan and Kapdan, 2006; Pratoomyot *et al*., 2005).Currently the microalgae biomass production is still in a developing phase and a lot of work is necessary to enhance the productivity and to reduce the production cost. The most challenging problems for the microalgae production industry include capital and operating cost, difficulties in controlling the culture conditions, contamination of bacteria or unwanted algae, unstable light supply and weather. Several strategies have been proposed to cope with these difficulties. First of all it is important to select a good microalgae/cyanobacteria strain that are rich in the target products, can tolerate temperature changes, high salinity and/or alkalinity. These strains can easily become predominant in the culture environment, thus greatly reducing contamination problems.

Identifying preferable culture conditions for improving the production as well as designing efficient and cost-effective microalgae cultivation systems are also critical points (Chen *et al*., 2011).In particular, the enrichment of different components (such as lipids, proteins or pigments) in microalgae biomass requires different cultivation conditions and operational strategies. Under stress conditions microalgae can change their metabolic pattern and strategies, in order to face the difficulties (Hu, 2004).

In this way microalgae are induced to synthesize and produce various secondary metabolites, modifying also the quantity of representative primary metabolites (fat, carbohydrate and protein).

Microalgae are very useful for the production of secondary metabolites some of them have particular interest because they constitute high-value products with several applications (Markou and Nerantzis, 2013).

However under stress conditions the decrease or the arrest of growth rates and consequently the decrease of the total production and productivity is observed. In some cases it is possible that the productivity of an accumulated compound cannot reach the productivity under regular conditions because of the decrease in the growth rates (Adams *et al*., 2013).This negative effect might be reduced applying a microalgae cultivation in multiple-stage process, in which in each stage optimum or appropriate conditions are adopted (Markou and Nerantzis, 2013). The topic of the optimization of a desirable compound under stress conditions is of particular significance and more research is needed.

Table 3. Functional ingredients from microalgae and technology production system

Production systems	Functional ingredients	Microalgae Species	Commercial products
Photobioreactors 	Astaxanthin	*Haematococcuspluvialis*	
Raceways and Ponds 	Phicobiliproteins	*Arthrospira maxima* *Arthrospiraplatensis*	
	β-carotene	*Dunaliella salina* *Dunaliellabardawil*	
	Polysaccharides: sulfatedpolysaccharide	*Chlorella* sp. *Porphyridiumcruentum* *C. pyrenoidosa*	
	Proteins	*Arthrospira maxima* *Arthrospiraplatensis*	
Fermenters 	Lipids PUFAs	*CrypthecodiniumcohniiNitzschialaevis* *Phaeodactylumtricornutum* *Monodussubterraneus* *Porphyridiumcruentum*	

1.5Main potential applications

Microalgae market is largely to be explored yet, although the use of microalgae as a food source or supplement has occurred for centuries (Spolaore *et al*., 2006). Nowadays, the utilization of high-value compounds derived from microalgae is restricted to only a few species of microalgae as summarized in Table 3.

The freshwater green algae *Chlorella* and *Scenedesmus*and especially the cyanobacteria *Arthrospira platensis* and *maxima* are preferred for the use in human food, animal and fish feed, partially because of their high protein content (50–60% of dry biomass) and nutritive value.Cyanobacteria, but also some green microalgae such as *Chlorella* and *Dunaliella*,showed an interesting polysaccharide fractions and are used as dietary supplements or pharmaceuticals (Shi *et al*., 2007).A few species of diatoms and dinoflagellata are a good source of long chain polyunsaturated fatty acids (LC-PUFAs) (Chen et al., 2007; Rocha *et al*., 2003).

Among the microalgae pigments, carotenoids and phycobiliproteins showed to be the most important pigments from a commercial food perspective (Curtain, 2000; Lorenz and Cysewski, 2000).

1.6Aim

Since many years several reports studied different microalgae compounds for their interesting application in food, feed and energetic sectors.

The main point of interest about microalgal compounds is the possibility to obtain very high concentrations of long chain polyunsaturated fatty acids (PUFAs), which are the most interest in food application, but also to obtain oil to energetic purpose.

Actually fish oil is the main source of PUFAs, but others possible sources can be bacteria, fungi and plants that are all currently being studied for commercial production. Unfortunately, fungi require an organic carbon source and usually show slow growth rate (Gunstone, 1996),and plants beside the need arable land, should be genetically modified to produce long chain PUFAs (Whitehead, 1985).

Instead, microalgae are the primary source of EPA and DHA in the marine food chain and can be grow fast under a variety of autotrophic, mixotrophic and heterotrophic culture conditions. FAO, 2012).The fatty acid content of numerous microalgae strains have been studied (table.2) (Worm, 2006; Hu *et al*., 2008).Lipid accumulation in microalgae usually occurs when they are cultivated under stress conditions. The major chemical and physical stimuli are temperature, light intensity, pH, salinity, mineral salts. However the most used strategy is the nitrogen starvation forcing microalgae metabolism towards lipid accumulation (Patil *et al*., 2007).

The aim of thisresearch was to investigate and modulate the lipid content and fatty acid profile of different microalgae strains and of the cyanobacterium*A. maxima*for food and animal feedapplications considering also an energetic purpose.

To achieve this aimdifferent objectives were pursued in the following Chapters.

Firstly various microalgae strains were selected on the basis of their lipid content and fatty acid profile by literature data.Subsequently parameters,known to influence the lipid content of microalgae, were studied and analyzed:

2. Production technologies (ponds, photobioreactors, small volumes): to select the best technologies for each strains in relation to their productivity, lipid production and fatty acid profile;
3. Growth media (wastewater, standard media,N starvation);
4. CO_2 and sodium bicarbonateadded:to improve the photosynthesis utilizing alternative source of C from industrial gas fuel;
5. Assessment of microalgae bioremediation capacity also to reduce the costs of fertilizers (usually amounting to the 20% of microalgae biomass production costs);
6. Compare the lipid content of microalgae with other vegetable oil to evaluate their possible use in food and feed industries.

The research was carried out at Criaq (Interdepartmental Research Center for management resources biological and Aquaculture -University of Naples, Federico II) and at the Laboratory of Marine Biology and Environmental Biomonitoring (Federal University of Baiha, Brazil).

The PhD thesis is organized in 6 chapters. First chapter is a general introduction, followed from 4 experiments reported as scientific papers, that are submitted or in submission and a final conclusion.

The four experimental chapters with the relative aims are:

Chapter 2: Growth of two strains of *Botryococcus braunii*in domestic effluents before and after pretreatment stage: productivity, FA-profiles and biodiesel quality.

The aim of this work was to evaluate the growth of two strains of *B. braunii* in secondarily treated sewage and to compare their bioremediation power, productivity, FA-profiles and fuel quality of the generated biodiesel.

Chapter 3: Effects of different environmental variables on amount and quality of lipid content of *B.braunii* cultivated in small and large scale.

The aim of this chapter was to evaluate, on a small and large volumes, the effects of relevant environmental operating variables (different grow condition) on *B. braunii* (SAG 38.01). The lipid content was studied in terms of quantity and quality, to define the best grow condition to maximize the per cent lipid production of dry weight, to obtain the fatty acid profile most interesting for agro-food application, without reduce excessively mode the biomass productivity.

Chapter 4: Strategies to improve lipid content and profile in *Phaeodactylum tricornutum***The aim of this research is to evaluate, on a small and large scale, the effects of relevant environmental operating variables (different grow condition) on *P. tricornutum* (SAG 10.901A). The lipid content was studied in terms of quantity and quality, to define the best grow condition to maximize the per cent lipid production of dry weight, to obtain the fatty acid profile most interesting for agro-food application without reduce in excessive mode the productivity of microalgae studied.**

Chapter5*:* Lipid content and fatty acid profile of *A. maxima* cultivated in south of Italy

The main purpose of this work is to evaluate the biomass productivity, lipid content and fatty acid profile of *Arthrospira maxima* cultivated in pond under greenhouse in South of Italy (Portici , Naples) in a period of production from June to November in two years - 2012 and 2013.

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Chapter 2

Growth of two strains of Botryococcusbrauniiin domestic effluents before and after pretreatment stage: productivity, FA-profiles and biodiesel quality.

2.1 Introduction

Microalgae, as an alternative and renewable source of biomass feedstock for biofuels, have been explored previously. Nevertheless, a scalable and commercially viable system has not yet been feasible. The general claims are that hydrocarbon content of algae, and specially fatty-acids, isoprenoids andtriacylglycerols (TAG), have the potential to compensate for future decline of crude oil production if algae growth and harvesting can be energetically and environmentally sustainable (Chisti, 2007; Metzger et al., 2003).

The use of appropriate technologies with increased efficiencies and reduced environmental impacts, by coupling biomass cultivation with CO2 mitigation (for carbon credits) and wastewater treatment (for nutrient removal) will lower production costs and provide additional benefits (Wijffels and Barbosa, 2010).

Industrialized countries generate a great volume of urban and industrial wastewaters. It is estimated that the volume of domestic effluents generated by North America, Europe and Latin America is of approximately 70, 63 and 47 km3y-1, respectively (Uno, 2009). These effluents should not be dumped directly into rivers, lakes or the sea before treatment to reduce contaminants to environmentally safe levels. Special attention is required for inorganic substances which encourage vegetal growth, such as ammonium, nitrates and phosphates, that contribute for the eutrophication of the water bodies receiving the effluents (Uno, 2009). Conventional treatment of municipal wastewater, based on primary settling and secondary biological processing (e.g. activated sludge or trickling filter), removes only a fraction of the nitrogen (40%) and phosphorus (12%) contained in the waste.

In order to improve the process, new methods (tertiary steps) and, consequently, additional costs are required (Carey and Migliaccio, 2011).The European Directive 6498/15/EC establishes a threshold of 10 and 1mg per liter for total N and P. Wastewater Treatment Plant (WWTP) effluents commonly show N and P values around 20 – 70 mg/l and 4 – 12mg/l, respectively(Carey and Migliaccio, 2011). Therefore, there is still a clear need for new developments and biological systems are often considered to be the ideal means for responding to such a demand (Rawat*et a*l., 2011). In fact a successive biological nitrification–denitrification step can be successfully adopted to remove the residual nitrogen (Lam and Lee, 2012) and phosphorus. Microalgae based systems have shown a high potential to assist with nutrient removal. The economic costs are, however, a primary concern once improved nutrients removal would require an overall increase in energy consumption of about 60–80% (Lam and Lee, 2012). Therefore, new systems should explore the combination of wastewater treatment with the production of renewable energy to offset final costs (Li*et al*., 2012; Park *et al*., 2011).

It is generally recognized that microalgae play an important role in the self-purification of natural waters (Chan*et al*., 2011). A complete tertiary process aimed at removing ammonia, nitrate and phosphate will thus be about four times more expensive than primary treatment. Microalgae cultures may offer an efficient solution to tertiary and quinary treatments due to the ability to use inorganic nitrogen and phosphorus for their growth (Richmond, 1986, Oswald, 1988b, Oswald, 1988c, Garbisu *et al*., 1991,Garbisu *et al*., 1993 and Tam and Wong, 1995). Their capacity to remove heavy metals (Rai *et al*., 1981), as well as some toxic organic compounds (Redalje *et al*., 1989), therefore, does not lead to secondary pollution. Amongst beneficial characteristics they produce oxygen, and have a disinfecting effect due to increase in pH during photosynthesis (Mara and Pearson, 1986 and De la Noüe and De Pauw, 1988).

Algae can be used in wastewater treatment for a range of purposes, some of which are used for the removal of coliform bacteria, reduction of both chemical and biochemical oxygen demand, removal of N and/or P, and also for the removal of heavy metals Teles *et al*. (2013).

Microalgae based systems have shown a high potential to remove nutrients from wastewaters simultaneously with the synthesis of valuable chemicals, besides carbohydrates and oils that can be used respectively for ethanol and biodiesel production. (Park *et al*., 2011).Biofuels have been advocated as a suitable option to replace fossil fuels (Cerón-García *et al*., 2013; Altieri, 2009). However, several social and environmental issues are associated with increasing land crops-based biodiesel production (Altieri, 2009), while microalgae based systems were identified as capable of overcoming both economic and ecological problems 83 (Wijffels and Barbosa, 2010; Amaro *et al*., 2011). In addition, microalgae systems can generate further commodities such as bio-kerosene, bio-plastics, bio-hydrogen, biogas and other chemicals derivatives (Sawayama *et al*., 1994; Chisti and Yan, 2011). *Botryococcus braunii* is a colonial Chlorophyceae (green microalga) which is widely distributed on all continents, in freshwater, brackish and saline lakes, reservoirs or even small pools, situated in temperate, tropical and continental zones as well. This species is characterized by an original organization of colonies and an unusual capacity to produce unsaturated long-chain hydrocarbons, reaching contents ranging from 15% to 75% of its dry weight. In addition, it produces extra-cellular polysaccharides that induce the formation of colonies, the size of which depends on the hydrodynamic stress within the bioreactor. Its great potential as a renewable source of basic or combustible chemical products has been demonstrated by different research groups(Li*et al*. 2012). This alga is, therefore, a potentially good renewable source of chemical feedstock or fuel (Largeau *et al.* 1980; Casadevall *et al*. 1985; Wolf *et al*. 1985a,b; Kojima and Zhang 1999). Both economic and technical barriers have prevented the commercial development of methods for production of hydrocarbons using *B. braunii*. (Sawayama *et al*. 1992, 1994) reported continuous culture of the hydrocarbon-rich microalga *Botryococcus braunii* in secondarily treated sewage. They examined the growth and hydrocarbon productivity of *B. braunii* on secondarily treated sewage in a continuous system, and reported the consumption of inorganic nutrients in this process. Also Orpez *et al.* (2009) utilized *B. braunii* to removal nitrogen and phosphorus from secondarily treated sewage from domestic wastewater and reported good performance in terms of nutrients removal efficiency and lipids production.

The aim of this work was to evaluate the growth of two strains of *B. braunii* in secondarily treated sewage and to compare their bioremediation power, productivity, FA-profiles and fuel quality of the generated biodiesel

2.2 Material and methods

2.2.1 Algal strain and culture conditions

Two strains of *Botryococcus braunii*(UTEX LB 572 and LABIOMAR/IB/UFBA IBL C115). were used in this work .The strain LABIOMAR/IB/UFBA IBL C115 is a local strain presently kept in the Microalgae Bank of the Marine Biology Lab (LABIOMAR) of Federal University of Bahia (Brazil). The inocula were axenically prepared using CHU-13 medium The strains were cultivated under constant agitation (90 bpm), aeration (with 2.5% CO_2 addition), under a photonic fluxof 174 $\mu E/m^2/s$ (fluorescent lamp) (photoperiod of 12:12h light:dark cycle) and at a constant temperature of 25±1°C.

2.2.2 Experimental set-up

The domestic effluents were collected after the pretreatment stage (physical removal of large particles and fat materials) and at the discharge from the aeration tanks. Samples were collected from a Wastewater Treatment Plant (WWTP) in the municipality of Salvador, Bahia, Brazil. The wastewaters were utilized no treated and the control was represented by CHU 13 medium (Largeau*et al*., 1985).
The experiments were carried out in triplicates using 1 liter borosilicate Pyrex flasks.

2.2.3 Analytical methods

Microalgae growth was directly monitored by optical density (OD $_{680nm}$), pH, temperature (^{o}C) and total suspended solids (g/L) (APHA – Standard Methods, 1995). Biomass was recovered by centrifugation (4.000 g at 10°C for 20 min) followed by freezying and lyophilization. The dry weight was estimated gravimetrically (APHA, Standard Methods, WPCF 1995). Dried biomass was also tested for total lipids (Folch *et al.,* 1957) as percentage of dry weight.

Samples of the wastewater effluents were analyzed for ammonium ($N\text{-}NH_4$), nitrate ($N\text{-}NO_3$), nitrite ($N\text{-}NO_2$), phosphate ($P\text{-}PO_4$), pH, conductivity, turbidity and total suspended solids (TSS) according to APHA, Standard Methods (1995).

The fatty acid composition analysis was performed by GC after derivatization to fatty acid methyl ester (FAME) with 2 N KOH in methanol, according to the IUPAC standard method (AOCS, 1998; Ruiz et al., 2008). FAME, including methyl octanoate, were analyzed on a Perkin Elmer AutoSystem XL gas chromatograph (Perkin Elmer, Waltham, MA, USA) equipped with a PTV(programmed temperature vaporizer), a flame ionization detector, and a capillary column 100 m _ 0.25 mm inner diameter, film thickness of 0.20 mm. Stationary phase 50%cyanopropyl methyl silicone (Supelco, USA). The carrier gas, helium, was introduced at a flow rate of 20 cm/s. The oven temperature program was as follows: 120°C for 5 min,5°C/min ramp to 165°C for 5 min; and then 10°C/min ramp to 240°C for 20 min. The split ratio was 1/60, and the flam ionization detector temperature was set at 260°C(Romano, 2013). Peaksidentification was obtained using the external standard Supelco TM 37 FAME mix (Supelco) by comparing the retention times with the pure standard components.

2.2.4 Growth kinetics and productivity

Kinetic parameters were estimated using a sigmoid model (Origin® v.7.0) as described by Nascimento et al, 2013. The software is equipped with a statistical package for testing the fitness of the model in describing the biological response. The results are expressed as probability ($P<0.05$). The kinetic parameters were also crosschecked using linear regression of the exponential phases. This approach was applied on the experimental data and their natural logarithmic (Ln) transformed values, for the calculations of productivity,

For statistical analysis it was also applied linear regression (with at least five points) for assessing correlations between variables (R2 available in the text). A parametric analysis of variance (ANOVA) was carried out in order to assess the differences among tested groups. As post-hoc test, it was used the Tukey's multiple comparison test (MCT). All analysis were carried out within the GraphpadInstat® software (v. 3, 2008), at the significance level of 5 % ($P <0.05$).

2.2.5 Fuel quality of the generated Biodiesels

For the transportation sector, jet-fuel and biodiesel are products that can be respectively obtained from biomass and oil, by catalytic cracking and transesterification of fatty-acids composing the triacylglycerol molecules. Triacylglycerols, ranging from 11-80% (w/w) in algal oil (Zemke *et al*., 2010), are the main components for biodiesel production (Knothe , 2005; Demirbas and Demirbas 2011) and the responsible ones for the biodiesel quality, which is defined by the fatty-acids composing the TAG molecules (Nascimento *et al*., 2013; Teles *et al*., 2013). As the algal species have diverse fatty acid compositions, biodiesels from algae have different fuel quality, defined by parameters such as cetane number (CN), which estimates the delay of ignition and combustion performance, iodine value (IV), representing the total unsaturation within a mixture of FAME, and the saponification value (SV), which is a measure of the average molecular weight of all the FA present in oil (Knothe, 2005; Nascimento et al., 2013). The Cold Filter Plugging Point (CFPP) calculated based on the Long Chain Saturation Factor (LCSF), specifies at which temperature biodiesel will clog filters and fuel lines (Meher *et al*., 2006). The higher the CFPP value, the more the biodiesel tends to clog filters and fuel lines at low temperatures. Oxidation Stability (OS) estimates the biodiesel's susceptibility to deterioration and is mainly related to the content of double-bonds in the component FAME (Nascimento *et al*., 2013). The higher the polyunsaturated methyl ester content, which can be predicted

based on the degree of unsaturation (DU) of the FAME chains, the higher will be the biodiesel oxidation potential (Meher *et al*., 2006).

Potential biodiesel quality was estimated using correlative models as described by Nascimento *et a*l., (2013), involving empirical equations previously applied to vegetable oils and tested for accuracy and predictive capacity (Krisnangkura, 1986; Ramos *et al*., 2009; Francisco *et al*., 2010). The models were applied to FAME-profiling of each algal strain,and treatments, aiming to relate the molecular structure of fatty acids with the quality of the biodiesel produced (Nascimento *et al.*, 2013). The CN estimate model (Eq. 2) involved two independent variables, chain length and degree of unsaturation of each component ester. Chain length was expressed by saponification (SV) that is inversely related to the esters' molecular weight, using Eq. 3, while the degree of unsaturation was expressed by the Iodine Value (IV) using Eq. 4 (Krisnangkura, 1986)

$CN=46.3+ (5.458/SV) - (0.225 \times IV)$.....Eq. 2

$SV= \sum (560 \times N) / M$ .....Eq. 3

$IV= \sum (254 \times D \times N) / M$ .....Eq. 4

whereD= double bonds; M= molecular mass; N= percentage of each FAME;

The DU (Unsaturation degree) was calculated based on Eq.5 by considering the amount of MUFA and PUFA present in the algal oil, in weight percentage (Francisco et al., 2010).

$DU=MUFA + (2 \times PUFA)$…….Eq.5

The CFPP (cold filter plugging point) was calculated using Eq.6 in correlation with the LCSF (long-chain saturation factor), estimated using Eq. 7. LCSF influences CFPP by weighing up the values of the longer FA chains (weight percentages) in order to reproduce their impacts on fuel cold flow properties (Francisco et al., 2010).

$CFPP = (3.1417 \times LCSF) – 16.477$ ……..Eq. 6

$LCSF = (0.1 \times C16) + (0.5 \times (C18) + (1 \times C20)+ (1.5 \times C22) + (2 \times C24)$…...Eq.7

The estimated properties for each microalgae-based biodiesel were presented as the average of the products of the calculated FAME values and their percentage in the mixture (Wijfel *et al* 2010, Metzger *et al* 2005). Further details are described by Nascimento *et al* (2013) .The fuel properties (CN, SV, IV and CFPP) of the algal biodiesels were compared between the strains and treatments.

2.3 Results and discussion

2.3.1 Wastewater characterization

The wastewater chemical composition is showed in Table 1.

In the influent and effluent, the N source was represented especially by NH_4, that was the most suitable form of N to be assimilated by microalgae, while nitrate was the only N source in the CHU medium. It has been proven that *B. braunii* consumes nitrogen quickly in ammonium form (Orpez *et al.*, 2009). The effluent presented a lower content of N and P; thus, the N concentration was increased by adding NO_3 until reaching the same CHU13 N concentration.

Tabela1: Chemical variables analyzed in the wastewater samples

	Influent	Effluent	CHU-13
Turbidity (NTU)	235.0	4.50	0.00
Salinity (‰)	4.00	3.00	0.00
pH	6.50	6.40	6.80
SS (g l^{-1})	0.25	0.01	0.00
DQO *in nature* (mg O_2l^{-1})	770.9	63.1	49.5
DQO filtered (mg O_2l^{-1})	386.4	30.2	-
NH_4 (mgN-NH_4 l^{-1})	22.87±0.02	7.85±0.03	-
NO_3 (mgN-NO_3 l^{-1})	2.09±0	2.50±0.03	20.0
NO_2 (mgN-NO_2 l^{-1})	0.02±0.06	0.07±0.10	-
Total N (mgl^{-1})	24.98	190.42	20.0
PO_4 (mgP-PO_4 l^{-1})	10.20±0.01	1.89±0.01	6.81
N/P ratio	2.44	8.91	2.93

The N/P ratio was an important factor in the microalgae growth and the influent presented a ratio very similar to CHU medium (≈2.93) while the effluent that had a ratio nearly double (5.54) respect to CHU medium, began 10.58 after the addition of NO_3 (Table 1). Mc Ginn et al. (2011) reported that the concentration of NH_3 and PO_4^- in secondarily treated wastewater adequate to support high productivity should fall into the range of 20-40 mg $l^{-1}$and 1-10 mg l^{-1}respectively.

2.3.2 Biomass production

In order to compare the performance of two strains of B. braunii (UTEX LB 572 and LABIOMAR/IB/UFBA IBL C115), on different media under the experimental conditions above described, growth curves were plotted (figure 1), with the graphs showing the values of biomass (as dry weight) versus time (in days). In all treatments, the microalgae showed a typical batch growth with an exponential phase of about 5 to 8 days during the 10 days of incubation (Figure 1). It was assumed that the results do not show a significant lag phase for any of the trials because the inocula were carefully prepared before each experiment (Figure 1). The slopes of the curves and the data of productivity (Table 2) suggested that the effluent and the influent were suitable for biomass production generating adequate daily productivity and growth rates (Table 2).

Table 2. Productivity evaluated for the two strains of *B. braunii* grown on different medium

Growth medium	Productivity ($mgl^{-1}d^{-1}$)	
	UTEX LB 572	LABIOMAR/IB/UFBA IBL C117
Influent	38.50±10.7	58±10.7
Effluent	44.53±4.30	54.13±10.0
CHU	64.5±6.36	44.86±5.86

The data reported in Table 3 show that daily productivity and growth rate of the two strains were not statistically different (P>0.05), although *B. braunii*IBL C115 showed values that tend to be higher than *B. braunii*LB572 in the influent as well as in the effluent. In CHU medium a daily productivity of 64.5 mg L^{-1} d^{-1} and growth rate of 0.27 was registered for *B. braunii*LB572, values higher than the others obtained using wastewater medium, while *B. braunii*BL C115 presented a lower daily productivity (44.86 mg L^{-1} d^{-1}) in comparison with influent and effluent, and a growth rate of 0.23 d^{-1}. The two strains showed for the two parameters analyzed values that were statistically different (P<0.05) with the UTEX strain productivity 1.4 times higher than the Brazilian strain in CHU medium.

Yoo *et al.* (2010) reported for *B. braunii* (UTEX LB 572) incubated at 25 ± 1°C with continuous illumination of 150 μmol m^{-2} s^{-1}, a productivity of 26.55 ± 7.66mg dw L^{-1} d^{1} with 10% CO_2 addition and 77mg L^{-1} d^{-1} with 5.5% CO_2 in CHU medium in 14 days. These values were lower than the obtained in the present work where the cultures have been enriched with 2.5% CO_2. The longer lag phase in response to the additional 10% CO_2 and lower pHin culturescould be the reason for the lower growth rate and daily productivity.

Our results about specific growth rate are in line with data of Yoshimura *et al*. (2013), which reported for different *B. braunii* strains values in the range from 0.07 and 0.5 d^{-1} with the lowest value for *B. braunii* LB572. These results strenght the possibility to reuse the nutrients presented in the municipal wastewater after the pretreatment stage (physical removal of large particles and fat materials) and at the discharge from the aeration tanks.

The used municipal wastewater contained high concentrations of ammonium after the pretreatment stage (physical removal of large particles and fat materials) as well as at the discharge from the aeration tanks; the total nitrogen (T-N) removal efficiency by the two strains was about 62% for LB572 and 61 and 65% for IBL C115 respectively when influent or effluent was used as medium while was 48 and 61% in the control (CHU medium).

Tabela 3: Kinetic parameters of both *Botryococcus* strains

	B. braunii (UTEX BL 572)			*B. braunii* (IBL C115)		
	Influent	Effluent	CHU-13	Influent	Effluent	CHU-13
X_O (mg SS·L^{-1})	148	145	135	178	173	163
X_M (mg SS·L^{-1})	854	673	904	802	665	728
R^2	0.99	0.99	1.00	1.00	1.00	0.98
p-value	0.01	0.01	0.00	0.00	0.00	0.01
μ (day^{-1})	0.12	0.15	0.27	0.15	0.16	0.23
Productivity (mg ·L^{-1}·d^{-1})	38.5	44.53	64.5	58	54.13	44.86
Lipid productivity (mg ·L^{-1}·d^{-1})	20±0.4	23±1.2	19±1.2	14±0.6	19±2.3	17±1.2
TN removalefficiency (%)	62.7	62	48	61.1	65	61
TP removal rate (%)	100	100	100	100	100	100
Exponential phase (days)	5	5	5	5	5	5

Lipid biomass content is reported in table 4.

Fatty acid methyl esters derived from vegetable oil and animal fat through transesterification process is referred as biodiesel. Not all lipids can be converted to FAME, the chemical ingredient of biodiesel. Therefore, measurement of FAME in algal biomass is a direct indication of amount of lipids suitable for biodiesel production. *Botryococcus braunii* is known to contain longer chain hydrocarbons (Banerjee *et al*., 2002).

Table 4: Fatty acids profile evaluated for both *Botryococcus* strains on three different media

Fatty acid (%)		B. braunii (UTEX LB 572)			B. braunii (IBL C115)		
		Influent	Effluent	CHU-13	Influent	Effluent	CHU-13
Butyric	C4:0	1.97	1.3	1.3	2.6	0.4	-
Caproic	C6:0	2.24	0.5	2.0	0.7	1.0	1.89
Caprylic	C8:0	1.93	0.2	0.2	-	-	1.62
Capryc	C10:0	0.46	0.2	0.2	1.6	0.3	0.92
Undecanoic	C11:0	0.95	-	-	-	-	-
Lauric	C12:0	-	-	-	1.6	-	-
Myristic	C14:0	5.71	7.4	6.9	9.5	3.2	5.9
Pentadecanoic	C15:0	0.29	0.5	0.4	0.9	0.4	0.39
Palmitic	C16:0	21.19	26.0	20.0	29.4	15.1	20.39
Heptadecanoic	C17:0	0.21	0.6	0.4	0.5	0.5	0.66
Stearic	C18:0	6.29	11.1	8.4	12.9	7.4	8.44
Arachidic	C20:0	0.19	-	0.2	-	-	0.50
Heneicosanoic	C21:0	0.52	0.5	0.5	-	0.5	0.48
Behenic	C22:0	8.31	4.4	4.1	1.0	9.2	5.68
Lignoceric	C24:0	4.99	-	7.5	-	3.2	2.89
Myristoleic	C14:1	-	-	-	0.3	0.2	-
Palmitoleic	C16:1	1.16	1.4	2.0	3.0	-	-
Cis-10 Heptadecanoic	C17:1	1.04	-	-	-	0.5	-
Elaidic	C18:1 n9t	1.06	2.1	1.4	-	1.1	1.16
Oleic	C18:1n9 c	28.46	33.4	30.1	30.7	41.2	38.34
Cis-11Eicosenoic	C 20:1	2.70	0.7	1.5	1.6	1.5	1.81
Linolelaidic	C18:2n6t	0.21	-	0.2	-	-	-
Linoleic	C18:2n6 c	3.12	1.9	1.9	-	2.5	2.35
γLinolenic	C18:3n6	0.73	-	0.9	-	-	0.92
Cis 8.11.14-Eicosatrienoic	C20:3n6	0.89	0.7	2.8	-	1.2	1.08
Arachidonic	C20:4n6	1.89	0.8	0.8	0.4	1.3	1.12
Cis-5.8,11,14,17-Eicosapentaenoic	C20:5n3	1.87	5.6	3.5	0.7	7.0	2.89
Cis-13,16-Docosadienoic	C22:2	1.62	0.5	2.0	-	3.2	1.46
Saturated (% FA/TL)		55.25	52.7	52.1	60.7	41.2	49.76
Monounsaturated (% FA/TL)		34.96	37.6	35	36.6	44.8	41.31
Polyunsaturated (% FA/TL)		10.33	9.5	12.1	1.2	13.2	9.82
FA Total (% biomass DW)		20	24	18	14	18	17

In this experiment, the accumulation of lipids in *B.brauni* (UTX-LB572) varied from 18% to 24% of the dry weight; and the respective range shown by B. *braunii*(IBL C115) are from 14–18%.
The wastewaters had a positive influence on lipid production on B.braunii (UTEX-LB572), with the best result, found for the use of wastewater effluent (24%), while no positive effect was achieved on the local strain (IBL C117). In fact, the lipid production was approximately the same of the algae grown on a standard medium but in this case there was a positive effect on the total production (Table.2) This represents an attractive solution for a cheap and unappreciated product as the amount of lipid was increased in effluent and influent. Table 4 shows the qualitative analyses of FAs (percentages of monounsaturated (MUFA), polyunsaturated (PUFA) and saturated fatty acids (SFA)). *B.braunii* (UTX-LB572) grown in wastewaters showed the highest ratio of lipid accumulation per biomass (30%); Other authors have observed a small but different result with different amounts of FAs in C16:0 and C18 isomer Hai-Linh Tran 2010. In B.(IBL C117) different results for all FAs have been observed (Nascimento *et al*. 2013). The biodiesel fuel quality depends on the results of the overall fatty acids composition present in the strain. For both strains, the condition that produced at the same time the highest FA productivity and MUFA concentration was the treatment with wastewater effluent.

Quality of Botryococcus strains-derived biodiesels cultivated in wastewater
Oil quality is an essential factor for the success of the algal-based biodiesel industry (Nascimento *et al*., 2013). Several countries have established biodiesel quality standards and guidelines to regulate biodiesel production, for example EN 14214 in Europe, ASTM D 6751-10 in the United States, RANP/2008 in Brazil, and similar guidelines for South Africa and Australia (Stansell *et al*., 2011). The main fuel quality parameters (CN, SV, IV, CFPP and OS) were used to compare the quality of biodiesels that could be generated from two strains of *Botryococcus* cultivated in influent and effluent wastewater in relation to a nutrient-sufficient growth medium (Chu-13). These parameters can be estimated by analyzing the molecular characteristics of FAME composing the biodiesels (Table 4). It has been reported (Nascimento *et al*., 2013) that biodiesels with long chain fatty acids and a low degree of unsaturation (DU) tend to have a good ignition quality (higher CN values), but they do not show good flow performance at low temperatures (CFPP). On the other hand, biodiesels with good CFPP can be achieved by short chain fatty acids and high DU (Stansell *et al*., 2011). Therefore, it is clear that balanced fatty acid distribution is critical to improving biodiesel quality.
Table 4 shows the FA-profiles determined for the focused *Botryococus* strains cultivated on different media. The FA composition of the intracellular lipids was represented by the acids with the chain lenghts from 4 to 24 carbon atoms. Oleic acid (C18:1w9c) dominates the FA-profiles, for both strains in all treatments (28.46 minimum and and 41.20 maximum in biomass % dw), the highest values obtained in the effluent growth medium. It has been followed in quantity by Palmitic (C16:0) and Stearic (C18:0), which are the ones which predominate in most of Chlorophyta microalgae. Among the monoenoic acids, besides the Oleic (C18:1n9c) there were present in all treatments in high amounts, another isomers such as C18:1n9t (Eladic), C16:1(Palmitoleic) and C20:1 (Eicosenoic) were present. The strains were also characterized by the presence of three C18 and four C20 polyenoic acids. Differently from the results obtained from *B. braunii* Kütz IPPAS H-252 and *B. braunii* isolate from Shira Lake (Volova *et al.* 2003), all the polyenoic C18 had double-bonds in the n6 position, which means that they have not been synthesized via a direct elongation of oleic acid as was shown by Templier *at al*., (1987). The occurrence of C 28:1n9, which is a taxonomic characteristic of *B. braunii* strain (Metzger *at al*., 1990) could not be detected by the analytical technique used in the present study. Nevertheless the highest content in Oleic Acid (C18:1n9c) in all the treatments suggests that the taxonomic classification is the same for both strains, even though some differences can be noticed in their respective FA-profiles.
The general dominance of SFA for both species (Table 4), followed by similar significant amounts in quantity by MUFA such as oleic (C18:1) may guarantee that the biodiesels generated from

these stocks in all treatments would comply (Table5) with the European standard for CN (minimum 51). Yet they would meet the American and Brazilian standards with minimum CN of 47 and 45, respectively (Nascimento *et al*., 2013; Stansell *et al*., 2011). The lower values of PUFA for all the treatments will provide relatively low DU values even though the local *B. braunii* showed a larger variation in response to different treatments, with a higher Oleic production in the effluent treatment (Table 5). As high DU values tend to decrease the ignition quality and increase susceptibility to oxidation (Knothe, 2005; 2008), DU in oils has to be limited. European standards for biodiesel stipulate that linoleic ester (C18:2n6) and polyunsaturated ester (≥ 4 double-bonds) content must be less than 12% (mol/mol) and 1% (mol/mol), respectively (Knothe, 2006, Stansell *et al*., 2011). In fact, both strains of *B. braunii*, in all cultivation conditions in this study, satisfy this criterion (Table 5).

Biodiesels with more SFA have the best oxidative stability. However, especially when the carbon chains are longer as happens in *Botryococcus* strains oils, the derived biodiesels will have a high melting point, and could crystallize at normal engine temperatures (Meher *et al*., 2002). This is defined as poor CFPP properties (Knothe, 2008). Despite the fact that these characteristics provide good ignition properties, they decrease the flow and enhance the chances for plugging filters and fuel lines. Comparatively, however, biodiesels with a predominance of MUFA content in oils, are the ones that best satisfy all standards regarding CN and still have good CFPP values (Nascimento *et al*., 2013).

Yoo *et al*. (2010) have already suggested that *Botryococcus braunii* was the most appropriate for biodiesel production, based on their oil productivity and CN value. However, it has been reported that there are very significant differences in composition between species of the same taxonomic group (Zhila *et al*., 2001), which could lead to variations in biodiesel quality. The oils from the *Botryococcus* strains in this study were mostly composed of SFA, followed by MUFA, especially represented by the oleic acid (Table 4). This was not the same results reported by Nascimento *et al*., (2013)whohasshowed that *B. braunii* UTEX produced 76.3% of oleic acid, almost double *B. terribilis* (a local strain). This difference in composition leads to an imbalance in saturation/unsaturation ratios and further reduces biodiesel quality. Oleic acid helps balance ignition quality and cold filter properties (Knothe, 2008; Nascimento *et al*., 2013). Biodiesel with higher oleic acid content than SFA does not normally cause polymerization during combustion. However, the long carbon chains of oleic acid increase the CFPP value and may cause the formation of agglomerates. In addition, a higher oleic acid content increases unsaturation that may decrease oxidation stability.

Even though the Chu-13 medium has provide lower values of CFPP when compared to both wastewater treatments, other cultivation conditions such as low light may have contributed for the higher production of longer-saturated chains FA, increasing CFPP and the possibility of clogging filters. This property, which limit the application of *Botryococcus*- derived biodiesel in cold climate regions, can be minimized by cultivation strategies or by mixing the oils from species with opposite characteristics

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Appendix 1

Sceening test to define the CO_2 concentration to added in the experiments with *B. braunii*

Introduction

Usha Tripathi *et al.* (2001) reported higher growth and carotenoid production in different microalgae enriched with 0.5, 1.0, and 2.0% CO_2 concentrations. It was also reported that different algae require different levels of CO_2 for their photoautotrophic adaptability (Tripathi *et al.* 2001). Therefore, different levels of CO_2 were studied for enhancing the biomass and other metabolites production in two strains of B. braunii.

Analytical methods

Microalgae growth was directly monitored through measures of optical density (OD 680nm), pH and dried weight. The biomass of *B. braunii* UTEX LB 572 and IBL C115was harvested by centrifugation (4.500 at 4°C for 5 min) followed by freezing and lyophilization. The dry weight was estimated gravimetrically (APHA, Standard Methods, WPCF 1995).

Statistical analysis

The results are expressed as mean ± SE (standard error) of three replicates. All data were performed by SPSS 17.0 for Windows (SPSS Inc., USA). One-way analysis of variance (ANOVA) was used to evaluate differences among the four CO_2 concentrations. A value of $P < 0.05$ was considered statistically significant ($P<0.05$).

Results

The Carbon Assimilation in the biomass of *B. braunii* IBLC-115, cultivated at different concentration of CO_2, was reported in Table 1 together with the biomass productivity and lipids data. *B.braunii* IBL, enriched with 2 % CO_2, showed a Carbon Assimilation with a value significantly lower than the others CO_2 concentration treatments. However the Carbon Assimilation evaluated at CO_2 concentration of 3-5-10 % (v/v) didn't show significantly differences (Table 1). The lipid content evaluated in the different CO_2 treatments followed the same behavior of Carbon Assimilation, while the productivity weren't significantly different.

Table 1:% Carbon Assimilated, biomass productivity and % lipid in *B. braunii* IBLC-115 growth at different concentration CO_2 (v/v)

Strains	% $C0_2$ (v/v)	C Assimilation (% dw)	Biomass productivity (mg L^{-1} day^{-1})	Lipid (% dw)
***B. baunii* IBL**	2	23±5[b]	39±3	22±6[b]
	3	31±1.5[a]	34±5	31±2[a]
	5	31±3[a]	33±4	32±2[a]
	10	24±6[ab]	33±3	33±1.5[a]

In Table 2 was reported the Carbon Assimilated in the biomass of B. braunii UTEX 572. The treatments showed not significantly difference in Carbon Assimilation, biomass productivity and lipid production (% w/w).

Table 2: % Carbon assimilated, biomass productivity and % lipid in *B. braunii* UTEX growth at different CO_2 (v/v) concentration

Strains	% CO_2	C assimilation (% dw)	Biomass productivity (mg L^{-1} day^{-1})	Lipid (% dw)
***B.braunii* UTEX**	2	18±5	50±3	26±6
	3	22±1.4	46±4	32±2
	5	23±4.2	40±3.5	33±2
	10	19±1	15±3	28±4

The increase in biomass yields and lipid content in those two strains of *B. braunii* in response to a CO_2-enriched atmosphere when compared with control cultures, in the present study suggested to insufflate carbon dioxide to 3% (v/v) for the next experiments

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Chapter 3:

Effects of different environmental variables on quantity and quality of lipid content of *B. braunii* cultivated in small and large scale

3.1 Introduction

Microalgae are photosynthetic microorganisms that are able to use the solar energy to combine water with carbon dioxide to create biomass. Because the cells grow in aqueous suspension, they have more efficient access to water, CO_2, and other nutrients.

The Relationship between cultivation conditions and biosynthesis of value-added compounds has been studied. Under stress conditions (sub- or supra-optimal conditions) microalgae can change their metabolic pattern and strategies, in order to face the difficulties. The biomass composition is influenced by this dynamic change, that acts on the metabolic strategy inducing a fluctuating of the relative compounds content of the biomass (Hu, 2004). Microalgae are induced to synthesize and produce various secondary metabolites, in addition modifying also the quantity of representative primary metabolites (fat, carbohydrate and protein). The identification of preferable culture conditions to improve the production as well as to designe efficient and cost-effective microalgae cultivation systems are critical points.

In this study it has been considered three strategies for lipid production from microalgae: nitrogen starvation, carbon dioxide supply and sodium bicarbonate addition.

Nitrogen, which generally accounts for about 7–10% of cell dry weight, is an essential constituent of all structural and functional proteins in algal cells. In general, microalgae have a limited ability to produce nitrogen storage materials when growing under nitrogen-sufficient conditions. Until cell nitrogen falls below a threshold value, photosynthesis still continues, albeit at a reduced rate.

N starvation is an efficient environmental pressure used to increase lipid accumulation (Stephen *et.al* 2011). The general principle is that when there is insufficient N for protein synthesis required for growth, excess carbon from photosynthesis is channeled into storage molecules such as triglyceride or lipid. The microalgae lipid content in this condition could be doubled or even tripled (Perez-Garcia *et al.* 2011, Gouveria *et al.* 2006), and a linear relationship between the N source concentration and the lipid content was observed (Marinho -Soriano *et al.* 2006).

In addition to the increase in total lipid content in microalgal cells as a result of cultivation in N depleted media, it was found that gradually the lipid composition from free fatty acid-rich lipid changes to mostly triglyceride-containing lipid (Carlucci *et al.* 1999).

Another method to improve the microalgae productivity and the production of lipids is the addition of carbon dioxide, because the microalgae can grow more rapidly and convert solar energy into chemical energy via CO_2 fixation.

CO_2 fixation by microalgae has been positively correlated with its cell growth rate and light utilization efficiency, since it involves photoautotrophic growth (Jacob-Lopes *et al*., 2009). Some of the physicochemical parameters that affect CO_2 fixation include: temperature, medium composition, pH, light intensity and CO_2 concentration (Ho *et al*., 2011).

Microalgae can fix CO2 from different sources which can be categorized as CO_2 from atmosphere, CO_2 from industrial flue gases and CO_2 in the form of soluble carbonates ($NaHCO_3$/Na_2CO). Many microalgae and cyanobacteria species can actively take up HCO_3^- from the external environment via transport across the plasma membrane into the cytosol and derive CO_2 from HCO_3 via the action of carbonic anhydrase maintaining a steady state flux to ribulose-1,5-bisphosphate carboxylase oxygenase for photosynthesis. Alternatively, extracellular carbonic anhydrase can catalyse the inter-conversion of HCO_3^-, and CO_2. Both mechanisms of HCO_3 utilization have been reported in phytoplankton (Colman and Gehl 1983, Raven 1991, Merrett *et al*. 1996, Nimer et al. 1997, Huertas and Lubian 1998, Bozzo et al. 2000, Young *et al*. 2001). Sodium bicarbonate has been used as a carbon source for the study of growth and biochemical composition of different microalgae species (Guiheneuf *et al*. 2008, Jayasankar and Valsala 2008, Guiheneuf *et al.* 2009, Sostaric *et al*. 2009, Pimolrat *et al*. 2010, Yeh *et al*. 2010) and has been shown

to stimulate triacylglycerol accumulation (Guckert and Cooksey 1990, Gardner *et al*. 2012). Moreover, different species exhibit different potentials to utilize HCO_3 from the culture media (Nimer *et al*. 1997, Huertas and Lubian 1998).

Botryococcus braunii is a colonial Chlorophyceae (green microalga) which is widely distributed on all continents, in freshwater, brackish and saline lakes, reservoirs or even small pools, situated in temperate, tropical and continental zones as well. This species is characterized by an original organization of colonies and an unusual capacity to produce unsaturated long-chain hydrocarbons, reaching levels ranging from 15% to 75% of its dry weight (Metzger and Largeau, 2005). In addition, it produces extra-cellular polysaccharides that induce the formation of colonies, the size of which depends on the hydrodynamic stress within the bioreactor. Its great potential as a renewable source of basic or combustible chemical products has been demonstrated by different research groups. This alga is, therefore, a potentially good renewable source of chemical feedstock or fuel (Largeau *et al*. 1980, Casadevall *et al*. 1985, Wolf *et al*. 1985, Kojima and Zhang 1999).

The aim of this chapter is to evaluate, on a small and large volumes, the effects of relevant environmental operating variables (different grow condition) on *B. braunii* (SAG 38.01). The lipid content was studied in terms of quantity and quality, to define the best grow condition to maximize the per cent lipid production on dry weight, to obtain the fatty acid profile most interesting for agro-food application, without reduce in excessive mode the biomass productivity.

3.2 Materials and Methods

Strain and cultivation

B. braunii SAG 38.01 (University of Goettingen) was selected for this experiment and was grown and maintained routinely in CHU13 medium (Largeau *et al*., 1985).

N starvation

B. braunii SAG 38.01 was cultivated in different condition: nitrogen starvation with air and with the addition to 3% (v/v) CO_2. The inoculum was axenically prepared using CHU-13 medium without nitrogen. The growth was conducted under photonic flux of 174 $\mu E/m^2/s$ (fluorescent lamp) (photoperiod of 24 h light), constant temperature of 25 ± 1 ° for 28 day. The cultures were carried out in continuous, two harvests were effectuated after 14 and 28 days.The experiments were carried out in triplicates using 5 liter borosilicate Pyrex flasks.

Bicarbonate addition

B. braunii SAG 38.01 was cultivated adding bicarbonate (NaHCO3) at 2 concentrations, corresponding to 3% and 6% CO_2 (v/v), both in batch and in continuous. The bicarbonate was added in batch at concentrations of 6 and $12gL^{-1}$ on the final volume (10L) and in continuous at daily concentrations of 0.214 and 0.419 gL^{-1} trough a peristaltic pump (ISMATEC IPC 8 channel – 42ml/h).

Tests were conducted under constant mixing and photonic flux of 174 $\mu E/m^2/s$ (24/24 h light), at a constant temperature of 25 ± 1 ° for 28 day. The biomass was harvested after 14 and 28 days.The experiments were carried out in triplicates using 10 liter plexiglass photobioreactors.

Photobioreactors

B. braunii SAG 38.01 was cultivated in batch in standard medium (CHU13), under constant mixing blowing air, light 26 MJ/m^2/die(photoperiod of 9:15h light:dark cycle - data collected from Ministry of Agriculture Forestry and Environmental) and at a water temperature of 26±5°C. The growth was carried out under greenhouse in triplicates using polyethylene photobioreactors of 120 L (low technology).

3.2.1 Analytical methods

Microalgae growth was directly monitored through optical density (OD 680nm), pH and biomass dry weight. Biomass smaller volumes (5-10 liters) was harvested by centrifugation (4.500 at 4°C for 5 min) and then freeze-dried, while the biomass of photobioreactors of 120 L was harvested by continuous centrifuge (4.500 rpm) preceded by a flocculation step with NaOH (1N) and then freeze-dried.
The dry weight was estimated gravimetrically (APHA, Standard Methods, WPCF 1995).

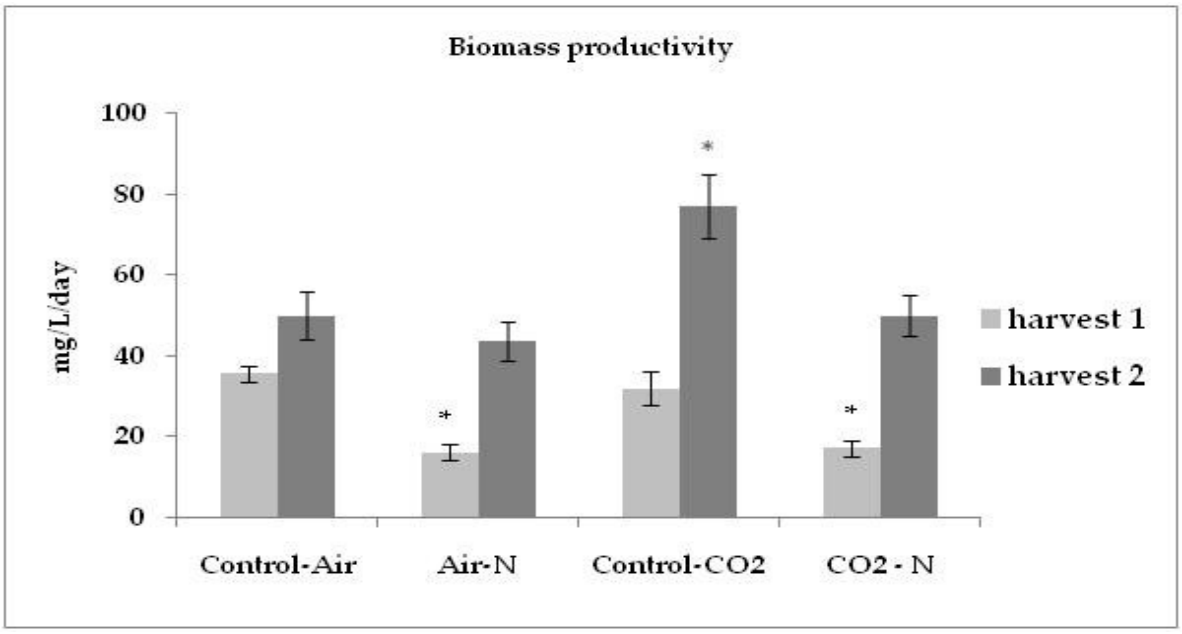
Lipid productivity and lipid profile

B. braunii 38.01 dried biomass was analyzed for total lipid content (Folch *et al*., 1957) and fatty acids profile.
The fatty acids profile was performed by gas chromatography after derivatization to fatty acid methyl ester (FAME) with 2 N of KOH in methanol, according to the IUPAC standard method FAME, including methyl octanoate.The analyses were carried out by Perkin Elmer AutoSystem XL gas chromatograph (Perkin Elmer, Waltham, MA, USA) equipped with a PTV(programmed temperature vaporizer), a flame ionization detector, and a capillary column 100 m- 0.25 mm inner diameter, film thickness of 0.20 mm. Stationary phase 50% cyanopropyl methyl silicone (Supelco, USA).
The carrier gas, helium, was introduced at a flow rate of 20 cm/s. The oven temperature program was as follows: 120°C for 5 min, 5°C/min ramp to 165°C for 5 min, and then 10°C/min ramp to 240°C for 20 min. The split ratio was 1/60 and the flam ionization detector temperature was set at 260°C (Romano *et al.* 2013). Peaks identification was obtained using the external standard Supelco TM 37 FAME mix (Supelco) by comparing the retention times with the pure standard components.

3.3 Results ad Discussion

Effect of nitrate starvation on biomass and lipid productivity of *B. braunii*

The lipids accumulation in many microalgae species occurs when they are under ''fattening'' conditions such as nitrogen limitation (Li *et al*., 2008, Mandal and Mallick, 2009).
However, it is necessary to explore how long the microalgal cells should be cultivated under N-starvation condition in order to obtain the optimal lipid production.
In this work *B. braunii* was cultivated under a high light intensity for 28 days without nitrogen and the lipid content and productivity was monitored.
The effects of N starvation on *B. braunii* biomass was reported in Graphic 1 while lipid content (% dry weight) and productivity (mg/l/day) are shown in table 1. After 14 days of cultivation (harvest 1) *B. braunii,* grown in CHU13 medium (control) with air or enriched with 3% CO_2 (v/v) presented similar biomass productivity (35.71 vs 30. mg/L/day, graph. 1). After 28 days (harvest 2) of cultivation *B. braunii* growth in CHU13 with 3% CO_2 showed the highest biomass productivity: 77 mg/L/day. The data were in line with Rao *et al.* (2007) results: at 1 and 2% level of CO_2 a significantly increase in biomass production was reported for different strains of *B. braunii* after a culture period of 20 days, but not before. In previous studies the biomass productivity of *B. braunii* showed not to be modified significantly with CO_2 addition at 10% v/v respect to the control culture mixed only with air (Chan Yoo *et al.* 2009).Meanwhile the lowest productivity was 16 mg/L/day obtained in nitrogen starvation condition without CO_2 addition after 14 days. After 28 days this value increased at 43.7 mg/L/day, resulting significantly different and lower than the value obtained for the control with CO_2 addition.



Graphic 1.*B. braunii SAG* Biomass productivity at 14 and 28 days (harvest 1 and 2 respectively) . Control-Air= CHU13+Air, Control-CO_2 = CHU13+3%(v/v)CO_2, Air-N= CHU13 without nitrogen +Air , CO_2-N= CHU13 without nitrogen +3%(v/v) CO_2.

The biomass productivity showed a significantly increase (P<0,05) after 28 days of culture in comparison to 14 daysfor all the treatments,

The ability to increase in biomass concentration during initial nitrogen starvation was observed for many microalgal strains (Silva *et al.*, 2009, Guarnieri *et al.*, 2011, Li *et al.*, 2008, Lu *et al.*, 2010, Msanne *et al.*, 2012, Packer *et al.*, 2011, Pal *et al.*, 2011, Pruvost *et al.*, 2009 and Pruvost *et al.*, 2011). Moreover it was expected that the lipid content during the stationary phase was higher than previous phase (Yoo *et al.*, 2010). The effects of nitrogen starvation on *B. braunii* lipid content (% dw) and productivity (mg/l/day) are reported in Table 1. With air mixing the lipid content in the two harvests (after 14 and 28 days) was higher than Control: 52% vs 22% and 65% vs 38% of dry weight, respectively.

Same trend was showed by the culture obtained by N starvation with 3%CO_2: higher values than control samples with CO_2 addition. The absence of nitrogen has been determined an increase in the production of lipids also in the experiment carried out by Shing (1992).

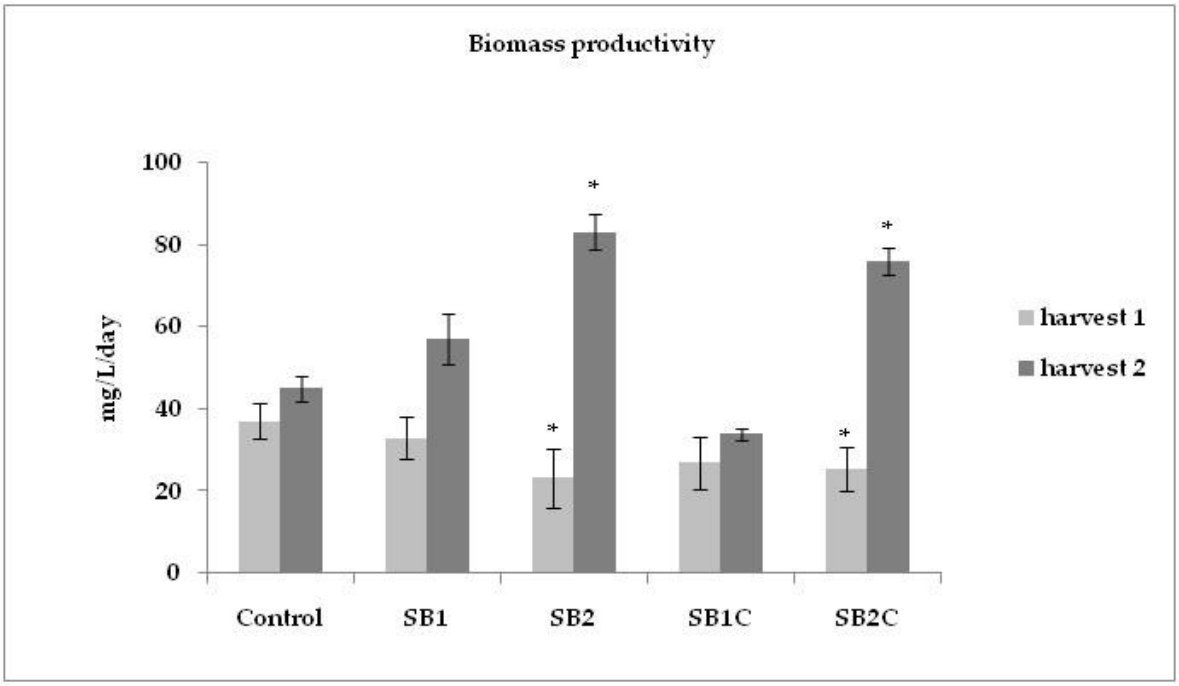
Table 1: Lipid content and productivity in *B. braunii* at 14 and 28 days (harv. 1 and 2)of cultivation on:CHU-13- Air (Control-Air), CHU13- 3% (v/v) CO_2 (Control -CO_2), CHU-13 without nitrogen - Air (Air-N), CHU-13 without nitrogen + 3%(v/v) CO_2 (CO_2-N).

	Harvest	Lipid Content (% dw)	Lipid Productivity (mg/ L/day)
Control-Air	I	22±5	7.7±1.9
	II	38±3.9	19±2
Air-N	I	52±7*	8.3±1
	II	65±4.3*	28.62±1.6*
Control CO_2	I	22±3	7.04±0.96
	II	40±7	31.3±4.8*
CO_2-N	I	37±5.1*	6.3±1
	II	58±6*	29±3*

The higher lipid percentage was obtained from nitrogen starvation culture with Air and CO_2, while the highest biomass productivity was observed in the Control culture with CO_2 added, the lipid productivity (mg/l/day) showed values not significantly different in the two treatment without N and in the control enriched with CO_2 3%(v/v) for the two harvests (Table 1).
Consequently, the CO_2 addition was identified as a critical treatment to achieve a high biomass productivity after 20 days, while nitrogen starvation as a critical treatment to achieve a high lipid content and productivity.
This result is consistent with previous reports, for example, the total lipid content of *Neochloris oleoabundans* and Chlorella sp. increases by a factor of 2 at a low N concentration (Hsieh and Wu 2009, Li *et al*. 2008a).
Data on *B. braunii* biomass and lipid productivity are relatively limited and evaluated for little volumes. Lee *et a*l. (2010) reported a biomass productivity of 35.7 mg/l/day and a lipid productivity of 11.5 mg/l/day of *Botryococcus* sp. cultivated in BG11 mixing with air for 7 days, while Yoo *et al.* (2010) for *B. braunii* UTEX 572 grown in CHU13 enriched with CO_2 10% (v/v), after a period of adaption, presented a productivity of 26.55 ± 7.66 mg/l/day (14 days cultivation) with a total lipid productivity of 5.51 ± 1.53. Mata *et al*.2010 in a review have reported for *B. braunii* a biomass productivity of 20 mg/l/day with a very huge variation in lipid content (25-75% dry weight biomass) without can define a realistic lipid productivity.

Effect of bicarbonate on biomass and lipid productivity of *B. braunii*
An adequate supply of inorganic carbon is essential forregular photosynthesis and growth in photoautotrophicmicroalgae. This may be achieved through the supply of gaseous CO_2 to the media in which the microalgae aregrowing, with high stripping leak. However, in commercial situations where the supplyof adequate CO_2 may be limited, alternative inorganiccarbon sources, e.g. bicarbonate salts (NaHCO3), could potentially be utilised. Furthermore, bicarbonate has greater solubility than CO_2, thus reducing issues associated with low retention times (Hsueh *et al.* 2007). However, interspecies differences in utilisation of bicarbonate as a carbon source may result in differences in metabolic efficiency and biochemical composition (Giordano *et al.* 2005).
In this research *Botryococcus braunii* after 14 days (I harvested) showed a biomass productivity statistically not different between Control and sodium bicarbonate added in batch at 6 g/L (37 mg/L/day and 33 mg/L/day, respectively), but after 28 days the biomass productivity incresead, reaching the highest values when sodium bicarbonate was used at concentration of 12g/l in batch and 0.429 g/L/day in continuous (Graphic 2) although the comparison was statistically not different.
White *et al*. (2012) instead, in seawater microalgae species showed that bicarbonate addition had significant effects on growth.The threshold level of inorganic carbon added to the cells, which

increase grow, is species dependent and will undoubtedly be intrinsically linked with light availability and other environmental conditions (Carvalho and Malcata, 2005).



Graphic 2. *B. braunii SAG* biomass yeld at 14 and 28 days (harvest I and II) *at* different level of $NaHCO_3$. Control=CHU13, SB1=CHU13+6 g/L $NaHCO_3$, SB2=CHU13+12g/L $NaHCO_3$,SB1C=CHU13 +0.214 g/L/day $NaHCO_3$, SB2C=CHU13 +0.428 g/L/day $NaHCO_3$.

Effect of $NaHCO_3$ addition on lipid production in *B. braunii* are showed in Table 2. For the first harvest (after 14 day) the highest lipid content of 31.5±4.2 % (dry weight) was obtained by the sample growth with sodium bicarbonate at 6 g/L (SB1). In the second harvest the highest lipid content was given by the control and sample SB1 (respectively 43.5±2.4 % and 40.3±2.7 %), both values not significantly different from SB2C. The lipid content increased significatively from the first to second harvest for all treatments, except for SB1C.

Dev Goswami *et al*. (2012) reported for *Selenastrum sp.* grown with sodium bicarbonate addition (from 20 to 100 ppm) a biomass productivity that increase to 1.1 mg/ml/day at 60 ppm of bicarbonate with a lipid content of 14% (dry weight): behavior not in agreement with the present study data for *B. braunii*.

Table 2. Effect of $NaHCO_3$ on lipid content (% on dry weight) and lipid productivity in *B. braunii* after 14 (harvest I) and 28 days of culture (harvest II). Control=CHU13, SB1=CHU13+6 g/L $NaHCO_3$, SB2=CHU13+12g/L $NaHCO_3$,SB1C=CHU13 +0.214 g/L/day $NaHCO_3$, SB2C=CHU13 +0.428 g/L/day $NaHCO_3$.

	Harvest	Lipid content (% dw)	Lipid productivity mg/ L/day
Control	I	20±3.1	7.42±1.06
	II	43.5±2.4	19.57±1.3
SB1	I	31.5±4.2*	10.3±1.7 *
	II	40.3±2.7	22.97±2.3*
SB2	I	11.5±3.4*	2.6 ±0.5*
	II	26.4±4.2*	21.9±1.8
SB1C	I	18.1±3.2	4.8±0.6*
	II	23.2±2*	7.8±1*
SB2C	I	13.3±3.7*	3.34±0.3*
	II	36.4±4*	27.6± 2.1*

It's important underline that the sample cultivated in sodium bicarbonate at 0.429 mg/L/day in continuous (SB2C) gave a good lipid productivity (27.6 mg/L/day), significantly different respect other samples (Table 2).

B. braunii cultivation in photobioreactors: biomass and lipid productivity

The microalgae massive culture for feed and food stocks is at the moment carried out in ponds (90% of the total biomass production) or photobioreactors (more expensive technologies, but more productive and safety). Large-scale algae production facilities are established in Asia, India, USA, Israel and Australia (F.A.O. 2008) and not data are presented for our latitudes.
Photobioreactors have been successfully used for producing large quantities of microalgal biomass (Molina Grima *et al.*, 1999, Tredici, 1999, Pulz, 2001, Carvalho *et al*., 2006), but often they present high cost management for the technologies associated.
To assess the variability in biomass production and lipid content on the basis of production technologies and environmental parameters the *B. braunii* production was carried out in photobioreactors (120L) of very low technologies and management cost, collocated under green-house in Portici (Naples). Biomass productivity (g/l/day), lipid content (% dry weight) and lipid productivity (mg/l/day) were evaluated. The lipid content was 33±5% on biomass dry weight while lipid and biomass productivity were 5.09±1 mg/L/day and 15.3±3.2mg/L/day, respectively; these data were significantly lower than small volume cultures (indoor with controlled temperature, irradiance and photoperiod). The different obtained productivity was probably because the high daytime light (see Mat and met paragraph) increased dissolved oxygen concentration that produced photo-oxidative damage to algal cells.

Fatty acid composition in *B.braunii* cultivated at different environmental conditions
The fatty acids composition of *B. braunii* is typical of Chlorococcales (Ahlgren *et al*. 1992), being very rich in palmitic acid, oleic acid, and linolenic acid.

Nitrogen starvation

The fatty acid profile (%) of *B. braunii* in nitrogen starvation is reported in table 3. The most important results can be summarized as follow: Palmitic acid (C16:0) and Stearic acid (C18:0) percentage did not change if the culture medium was with or without nitrogen while Oleic acid (C18:1) was higher in nitrogen starvation samples (Air-N, CO_2-N) and Linolenic acid was higher in control samples (Control-Air, Control CO_2). These results were generally compatible with

previous observations on the aging of nitrogen-starvation cultures (Khozin-Goldberg *et al.* 2002), which are characterized by an enhanced proportion of oleic acid and total fatty acid (TFA) content. The increase in oleic acid proportion during aging is believed to be, at least partially, the result of a shift from ω-3 to ω-6 unsaturated fatty acid in lipids that ensues after the transfer from the logarithmic to the stationary phase, as previously observed in many microalgae (Khozin-Goldberg *et al.* 2002). It has also already been shown that the TFA content and composition change during culture growth, however, nitrogen starvation induces a larger increase in the fatty acid (FA) content than the culture growth itself (Khozin-Goldberg *et al.* 2002). When decreasing the nitrogen concentration in the Chu-13 the oleic acid production was increased according with literature data (Gang-Guk Choi *et al.* 2010).

γ-Linolenic acid percentage showed no significantly difference between sample cultivated with CO_2 addition (Control-CO_2) and samples with nitrogen starvation (Air-N,CO_2-N) but resulted higher respect the sample cultivated in Air (Control-Air).The fatty acids concentrations didn't show significantly difference between the two harvests. The mean highest values omega- 6 (55%) are present in *B.braunii* cultivated with CO_2 (Control-CO_2).

Table 3. Fatty acid profile of *B. braunii* at 14 and 28 days of culture (harvest I and II). Control-Air= CHU13+Air, Control-CO_2 = CHU13+3%(v/v)CO_2, Air-N= CHU13 without nitrogen +Air , CO_2-N= CHU13 without nitrogen +3%(v/v) CO_2.

		Fatty acid profile (%)				
	Harvest	Palmitic	Stearic	Oleic	Linoleic	ɣ Linolenic
Control-Air	I	19±1.5	1±.3	15±1	24±2.3	8±2
	II	22±2.3	2±0.2	17±1.3	22±2.4	11±2
Air-N	I	20±2	3±0.9	36±3.2*	16±1*	22±2*
	II	21±1.7	2±0.5	34±3.2*	18±1.3	23±2*
Control CO_2	I	19±2	2±0.4	19±1.3	26±2.3	28±3*
	II	23±2.5	1±0	22±2*	24±2.2	29±2.5*
CO_2-N	I	20±2	2±0.2	32±3*	20±1.5*	25±2*
	II	21±1.8	2±0.3	32±0.5*	16±1.7*	24±3*

Sodium bicarbonate

The fatty acid profile of *B. braunii* cultivated at different concentrations of sodium bicarbonate are shown in table 4.It was found that *B. braunii* cultivated in medium enriched with 0.214 mg/L/day of sodium bicarbonate (SB1C-2 harvest) determined the highest value of oleic acid ($P<0.05$) and palmitic acid ($P<0.05$) according to Fang *et al.* (2004) Dickson *et al.* (2008) reported that the amount of Oleic acid 18:1 on a dry weight increased when the CO_2 concentration was raised from 1 to 30% (v/v),according data of the present experiment.Respect to the control, the γ-linolenic acid showed a statistically significant increase in all treatments, except in *B. braunii* cultivated in medium at 0.214g/L of sodium bicarbonate (SB1).The highest mean value of omega- 6 (48 %) was obtained from sample cultivated at 0.428 mg/L/day of $NaHCO_3$ (SB2C) showing in particular, a concentration of 37% of γ-linolenic acid .

Table 4. Effect of $NaHCO_3$ on fatty acid profile of *B. braunii* at 14 and 28 days (harvest I and II). Control=CHU13, SB1=CHU13+6 g/L $NaHCO_3$, SB2=CHU13+12g/L $NaHCO_3$,SB1C=CHU13 +0.214 g/L/day $NaHCO_3$, SB2C=CHU13 +0.428 g/L/day $NaHCO_3$.

		% Fatty acid profile				
	Harvest	Palmitic	Stearic	Oleic	Linoleic	γ-Linolenic
Control	I	16±2	12±3	11±2	20±1.9	7±2
	II	17±3	6±1	10±2.4	18±0.8	9±2
SB1	I	20±2.5	10±1.5	14±2.3	11±2.4*	24±4*
	II	31±5*	24±4*	24±4*	4±0.8*	6±1.5
SB2	I	14±3.2	3±1.2*	14±2.1	19±2.1	21±3*
	II	17.5±4.4	3.1±1*	29.9±6*	17±3	21.6±4*
SB1C	I	13±4	1±02*	13±3.2	20±4.2	14±3.1*
	II	24±3.2*	4±1	43±9.3*	9±2.2*	14±2.1*
SB2C	I	24±3*	4±1	16±3	25±3	19±2*
	II	12±2	5±1	11±2.2	19±3	37±4*

Photobioreactors

In table 5 fatty acid profile (%) of *B. braunii* grown in photobioreactors of 120 liters under green-house is reported. The fatty acid profile in this experiment is comparable with data obtained in little volumes (10L, Control-Air) (see Mat and Met paragraph). The predominant component of *B. braunii* grown in photobioreactors was palmitic acid followed by oleic acid, linoleic acid and γ-linolenic acid.The decrease in the unsaturation level of fatty acids as evidenced in this case, is well known during the temperature shift in a wide range of living cells (Sato and Murata, 1981).

Table 5: %Lipid profile in *B. braunii* at 14 days (harv. 1) in CHU-13 medium

Fatty acid	% dry weight
Palmitic	29±3
Stearic	2±0.3
Oleic	18±1.2
Linoleic	10±1
γ-Linolenic	9±1

Food and Feed application

Moreover, *B.braunii* lipid profile should be compared with that of vegetable oils. As it is shown in table 6 *B.braunii* linoleic acid profile is sometime comparable to that of vegetable oils used in human food and for animal feed, while *B. braunii* γ-Linolenic acid concentration shows values higher than vegetable oils. However, linseed γ-Linolenic acid profile is higher than that of *B.braunii*.

Due to the high presence of γ-linoleic acid, important for its anti-inflammatory and immune-modulating activity, and due to the presence of polyunsaturated fatty acid, a lot of application in human food and animal feed could be developed as functional ingredients.

A food ingredientis considered "functional" if, besides its nutritious capacity, it has a scientifically proven benefit for one or more functions of the human organism, improving the state of health or well-being or reducing the risk of disease.In some countries (Germany, France, Japan, USA, China, Thailand), food and feed companies have already started to market functional foods containing microalgae and cyanobacteria. Food safety regulations for human and animal consumption are the main constraint for the biotechnological exploitation of microalgal resources, however successful cases already exists. In 2002 the use of the marine diatom *Odontella aurita* by Innovalg (France) as a novel food was approved, following EC Regulation 258/97. Currently some microalgae-related health claims were evaluated by EFSA: among them the most interesting regarded *Chlorella pyrenoidosa* for digestive and liver health and *Spirulina* to improve glucose management.A series of claims regarding eye health, oxidative balance, cardiovascular system and connective tissue and joints for *H. pluvialis* astaxanthin were recently rejected, however they will be likely resubmitted soon.

Table 6: fatty acid composition of some vegetable oils used for human and animal nutrition (Zambia, 2007).

Acid		Borage	Canola	Cornusa	Coconut	Cottonseed	Linseed	ExtraVirOlive	Palme	Peanut	Rice	Soybean	Sun flower
Caprylic	C8:0				6.38								
Capric	C10:0				5.56								
Lauric	C12:0	0.06			45.46								
Myristic	C14:0	8.75	0.06		18.82	0.77	0.05		1.12	0.03	0.21	0.06	0.06
Palmitic	C16:0	0.06	3.75	10.34	10.08	21.87	4.81	8.7	42.7	9.4	16.9	9.9	5.7
Heptadecanoic	C17:0	3.46	0.04	0.07		0.08	0.05	0.17	0.11	0.12		0.1	0.04
Stearci	C18:0	0.22	1.87	2.04	4.31	2.27	3.03	3.47	4.55	2.65	1.78	3.94	4.79
Arachidic	C20:0	0.3	0.64	0.44	0.08	0.026	0.02	0.46	0.39	1.38	0.61	0.41	0,3
Bhenic	C22:0	0.11	0.35	0.31		0.36		0.13	0.58	3.14	0.77	0.48	1.16
Lignoceric	C24:0	12.96	0.27	0.26		0.12	0.01	0.05	0.06	1.66	0.41	0.21	0.31
Palmitoleic	C16:1	0.29	0.21			0.47		0.51		0.06		0.08	
Heptadecanoic-cis	C17:1	0.07				0.11	0.12	0.25	0.06	0.01		0.08	0.06
Oleic	C18:1	16.52	62.41		25.54	16.61	21.42	76.34	39.37	48.71	40.5	21.35	15.26
Linoleic	C18:2	38.47	20.12		59.27	56.35	15.18	8.64	10.62	31.06	36.2	56.02	71.17
y-Linolenic	C18:3	0.22	8.37		1.07	0.33	54.24	0.75	0.21	0.23	1.6	7.15	0.45
cis-11 Eicosenoic	C20:1	4.4	1.54		0.37	0.14	0.4	0.34	0.17	1.43	0.53	0.22	0.22
cis -11-14Eicosadi	C20:2	0.5	0.11		0.09	0.1	0.39				0.11		0.09
Euric	C22:1									0.12			
cis 13-16-Docosano	C22:2	2,09						0.19					
Nervonic	C24:1		0.26		0.2	0.16	0.1		0.06		0.38		0.39

3.4 Conclusions

This study demonstrates that lipids content and productivity of *B.braunii* was significantly enhanced using treatmentsas nitrogen starvation with and without CO_2, and using as C source sodium bicarbonate (addition in continuos and in batch). The sodium bicarbonate can be considered a possible substituteof CO_2 for *B. braunii*, taking into account that the $NaHCO_3$ is more stable source of C respect to CO_2 gas (reduction in leak). The massive production of B. braunii in low cost and technology photobioreactors showed values for biomass productivity and lipid content lower respect to the other experiments in small volumes, because submitted to more stress factors dependent on the change in the environmental temperature and photoperiod, but resulting economically sustainable.

The data on lipid content and lipid and biomass productivity were in line with literature data. The fatty acid profile of *B. braunii* resulted very interesting for application in food and feed industries, being characterized by some very important fatty acid as Palmitic, Stearic, Oleic, Linoleic and ◎-Linoleic, which composition and quantity can be modulated applying different environmental culture conditions. *B.braunii* lipid profile can be compared with vegetable oils, linoleic acid profile is sometime comparable to that of vegetable oils used in human food and for animal feed, while *B. braunii* ◎-Linoleic acid concentration shows values higher than that of vegetable oils.

Due to the high presence of ◎-Linoleic acid, important for its anti-inflammatory and immune-modulating activity, and due to the presence of polyunsaturated fatty acid, a lot of application in human food and animal feed could be developed.

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Chapter 4

Strategies to improve lipid content and profile in *Phaeodactylum tricornutum*

The culture of marine microalgae for the production of polyunsaturated fatty acid (PUFA) has become a subject of interest in recent years, but it has not yet reached commercial scale. The only currently available commercial source of n-3 PUFA is fish oil, but the amount of fish oil is continuously decreasing while the demand for PUFA is continuously on the increase. Microalgae, are a natural source of long-chained, polyunsaturated essential fatty acids such as EPA and DHA. Microalgae are photoautotrophs oraganisms using light energy to produce chemical energy and convert inorganic carbon (CO_2) into sugars and organic compounds. Few algae species are used commercially today, also for the industrial production of essential oils (e.g. *Schizochytium* sp. And *Crypthecodinium cohni*). The important parameter for commercial scale cultivation is productivity, given as the biomass produced per volume or area over time. The availability and supply of energy and nutrients affect the biomass and lipid productivity and the EPA/DHA content in the cell. Some species can accumulate > 50% lipids/DW under stress conditions, and the aim is to optimize the fraction in EPA/DHA of these lipids.

Photoautotrophic microalgae can produce 15-30% EPA of total fatty acids.

Because the cells grow in aqueous suspension, they have more efficient access to water, CO_2, and other nutrients. Very interesting is the relationship between cultivation conditions and the biosynthesis of value-added compounds. Under stress conditions (sub- or supra-optimal conditions) microalgae can change their metabolic pattern and strategies, in order to face the difficulties. The biomass composition is influenced by this dynamic change, that acts on the metabolic strategy inducing a fluctuating of the relative compounds content of the biomass (Hu, 2004). Microalgae are induced to synthesize and produce various secondary metabolites, in addition modifying also the quantity of representative primary metabolites (fat, carbohydrate and protein). Identifying preferable culture conditions for improving the production as well as designing efficient and cost-effective microalgae cultivation systems are critical points.

In this study have been considered two strategies to increase lipid production from *P. tricornutum*: carbon dioxide addition and nitrogen starvation.

N is an essential constituent of all structural and functional proteins in algal cells. In general, microalgae have a limited ability to produce nitrogen storage materials when growing under nitrogen-sufficient conditions. Until cell nitrogen falls below a threshold value, photosynthesis still continues, albeit at a reduced rate.

N starvation is an efficient environmental pressure used to increase lipid accumulation (Stephen *et.al* 2011). The general principle is that when there is insufficient N for protein synthesis required for growth, excess carbon from photosynthesis is channeled into storage molecules such as triglyceride or lipid. The microalgae lipid content in this condition could be doubled or even tripled (Perez-Garcia *et al.* 2011,Gouveia *et a.l* 2006), and a linear relationship between the N source concentration and the lipid content was observed (Marinho-Soriano *et.al* 2006). In addition to the increase in total lipid content in microalgae cells as a result of cultivation in N depleted media, it was found that gradually the lipid composition from free fatty acid-rich lipid changes to mostly triglyceride-containing lipid (Carlucci *et.al* 1999). Another method to improve the microalgae biomass productivity and the production of lipids is the addition of carbon dioxide,

because the microalgae can grow more rapidly and convert solar energy into chemical energy via CO_2 fixation. CO_2 fixation by microalgae has been positively correlated with its cell growth rate and light utilization efficiency, since it involves photoautotrophic growth (Jacob-Lopes *et al.*, 2009). Some of the physicochemical parameters that affect CO_2 fixation include: temperature, medium composition, pH, light intensity, and CO_2 concentration (Ho *et al.*, 2011). *Phaeodactylum tricornutum* (Bohlin 1897) is a unicellular brown diatom penate, belong class Bacillariophyceae. It is the only species in the genus *Phaeodactylum*, distributed in same European countries, America ,Australia and New Zealand, Asia and Pacific Island. The most notable characteristic of this strain were its ability to produce a high proportion of fatty acids reaching contents ranging from 18% to 57% of its dry weight (Mata.*et a.l* 2010) . EPA can account for up to 20–40% of the total fatty acids, as a function of culture conditions (Ibáñez González *et al.* 2000). EPA is an essential fatty acid for the human metabolism and is involved in the blood lipid equilibrium, lowers triglyceride levels in blood serum, reduces the degree of platelet aggregation, is anti-inflammatory ,and prevents hypertriglyceridemia and various carcinomas . From a down-stream processing perspective, this strain has an additional advantage in effect it produces little quantity of DHA. Thus, the potential problem of separating DHA from EPA does not arise. (Yongmanitchai *et al.*1991).
The aim of this research is to evaluate, on a small and large scale, the effects of relevant environmental operating variables (different grow condition) on *P. tricornutum* (SAG 10.901A). The lipid content was studied in terms of quantity and quality, to define the best grow condition to maximize the per cent lipid production of dry weight, to obtain the fatty acid profile most interesting for agro-food application without reduce in excessive mode the productivity of microalgae studied.

4.1 Materials and Methods

Strain

The species *P. tricornutum* (SAG 10.901A), obtained from University of Goettingen, was selected for this experiments and cultivated at University of Naples *Federico II* (Criacq). The strain was grown and maintained routinely in 1/2 SWES medium, in standard condition (temperature of 23 ± 2 C°, under photonic flux of 174 μE/m²/s - fluorescent lamp, photoperiod of 24 h light). The inocula were axenically prepared.

Cultivation indoor (little volumes and photobioreactors: N starvation

P. tricornutum was cultivated at different conditions: nitrogen starvation without CO_2 addition and with 3% (v/v) CO_2.
N starvation condition was applied gradually (semi-continuus), after the exponential medium phase was reached, a specific microalgae culture volume was harvested and the harvested culture volume was replaced with the same volume of N-depleted medium. The nitrogen content in the medium was checked during the experiment using the nitrate assay kit (HATCH Metod 8507).

The experiment (conducted in triplicate) was carried out in little volumes using 10 liter borosilicate Pyrex flasks and in a column bubble photobioreactor (250 L) of PMMA at light and temperature controlled.
The microalgae growth in little volumes was monitored for 28 days, under a photonic flux of 174 μE/m²/s, a photoperiod of 24 h light, constant temperature of 25 ± 1 °C and under constant air mixing. Every 7 days 2,5 L of culture were collected.

The experiments in photobioreactor were conducted for 21 day, under light (1W/L neon lamp, they light Philips), with a photoperiod of 24 h light, temperature of 21 ± 2 °C and under constant air mixing , every 7 days 165 L of culture were collected.

Cultivation outdoor (photobioreactors and ponds)

P. tricornutum was cultivated in standard medium, under constant air mixing, in photobioeactor (May 2013) and ponds (July 2013) outdoor under green-house.
The environmental conditions for photobioreactor culture were: light 26 MJ/m^2/die, photoperiod of 9:15 light:dark cycles (data of Ministry of Agriculture Forestry and Environmental), temperature water grave of 26±2°C.
Photobioreactor: the experiments were carried out in triplicate for 14 days under greenhouse, using a low technology photobioreactor (120 L) of polyethylene. The harvest was done in batch.
The environmental conditions for pond culture were: light 25 MJ/m2/die, photoperiod of 10:14 light: dark cycles (data of Ministry of Agriculture Forestry and Environmental), average temperature water grave of 23±2 °C.
Pond:the experiments were carried out for 21 days under greenhouse, using a low technology pond (2.5 m^3). The harvest was done in semi-continuous. For coating the pond have been used low-cost materials, such as polyethylene for food use.

4.1.1 Analytical methods

The microalgae growth was directly monitored by optical density (OD 625 nm), pH, temperature (°C). The harvesting was carried out flocculating the culture with NaOH (5 N) until a pH of 10.4, and subsequently centrifuged in a continuous centrifuge at 4.500 rpm for 5 min. The wet biomass was dried by freeze-drying and lyophilization. The dry weight was estimated gravimetrically (APHA, Standard Methods, WPCF 1995).

Determination Lipid productivity and lipid profile

Dried algal biomass was analyzed for total lipid content (Folch *et al*., 1957) as percentage of dry weight. The fatty acids profile was performed by gas chromatography after derivatization to fatty acid methyl ester (FAME) with 2 N of KOH in methanol, according to the IUPAC standard method . FAME, including methyl octanoate, were analyzed on a Perkin Elmer AutoSystem XL gas chromatograph (Perkin Elmer, Waltham, MA, USA) equipped with a PTV (programmed temperature vaporizer), a flame ionization detector, and a capillary column 100 m -0.25 mm inner diameter, film thickness of 0.20 mm. Stationary phase 50% cyanopropyl methyl silicone (Supelco, USA).
The carrier gas, helium, was introduced at a flow rate of 20 cm/s. The oven temperature program was as follows: 120°C for 5 min, 5°C/min ramp to 165°C for 5 min, and then 10°C/min ramp to 240°C for 20 min. The split ratio was 1/60 and the flam ionization detector temperature was set at 260°C (Romano *et al.* 2013). Peaks identification was obtained using the external standard Supelco TM 37 FAME mix (Supelco) by comparing the retention times with the pure standard components.

4.2 Results and Discussion

Cultivation indoor (little volumes and photobioreactors): N starvation

The lipids accumulation in many microalgae species occurs when they are grown in nitrogen-depleted cultures (Li *et al*., 2008Lombardie Wangersky , 1991, Mc Ginnis *et al.* , 1997). Several

recent reports (Mandal and Mallick, 2009) demonstrated that the effects of nitrogen deficiency was a sharp increase in the lipid content, because the condition of nitrogen-depletion probably tends to transforms carbon protein or peptides to lipids or carbohydrates. However, it is necessary to explore how long the microalgal cells should be cultivated under N-starvation condition in order to obtain the optimal lipid production.

In this work *P. tricornutum* was cultivated under a high light intensity for 28 days without nitrogen ,and the lipid content and productivity was monitored.

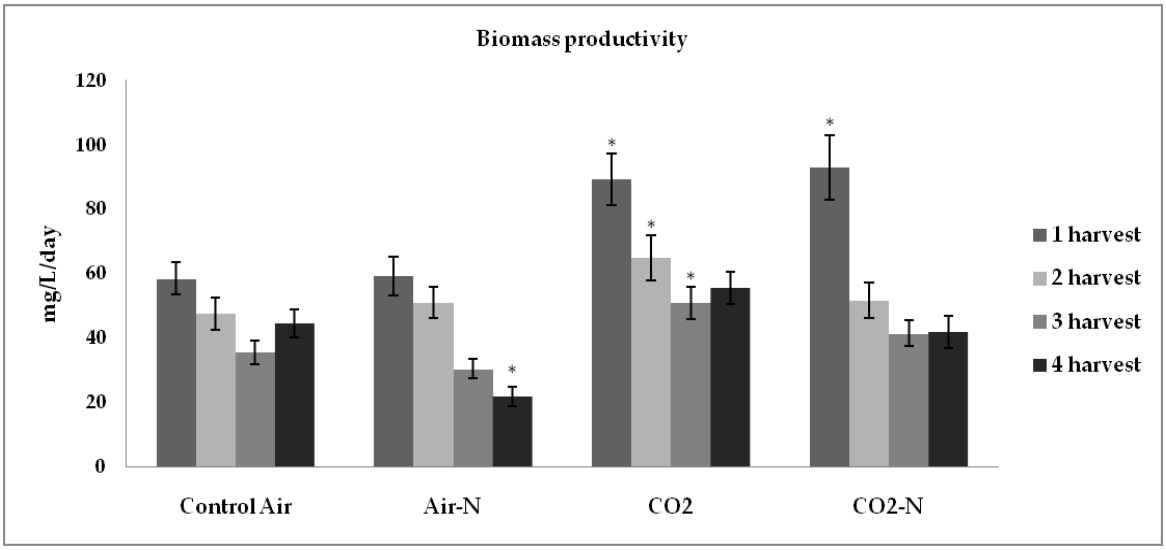
The effects of N starvation on *P. tricornutum* biomass was reported in Graphic 1 (small volumes) and Graphic 2 (photobioreactor), while lipid content (% dry weight) and productivity (mg/L/day) are shown in table 1 (little volumes) and table 2 (photobioreactor).

Phaeodactylum cultivated in flaskes with ½ SWES with CO_2 (3% v/v), and CO_2 3% without nitrogen (CO_2-N) after 7 days of cultivation (harvest 1) presented the highest biomass productivity: 89.4 mg/L/day, and 93 mg/L/day respectively, values significantly different from the culture growth in ½ SWES medium enriched with air (Control-Air) and 1/2 SWES medium nitrogen-depleted (Graphic 1).At 14 and 21days (harvest 2 and 3) high biomass productivities were obtained in culture cultivated with 1/2 SWES medium and CO_2 3% (v/v) with values respectively of 65 mg/L/day and 51 mg/L/day although not significantly different to the other treatments.

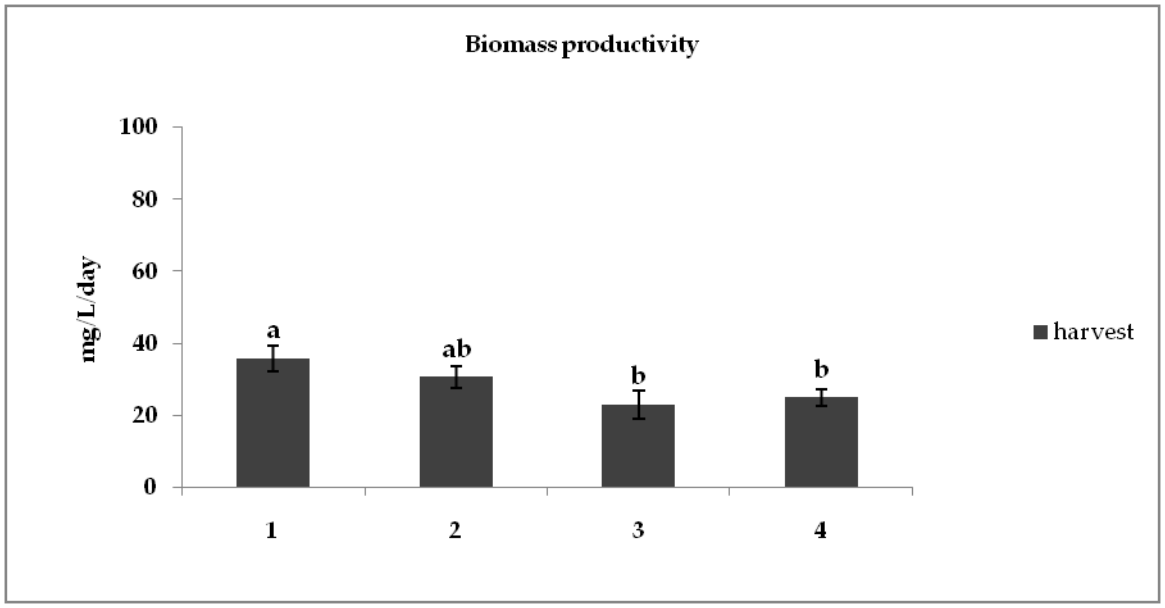
The lowest biomass productivity (22 mg/L/day) was registered at the last harvesting (after 28 days) of *P. tricornutum* growth in standard medium with air mixing and withot nitrogen.

Although on all harvested the medium (with and without nitrogen) was reintegrated, the productivity was different,Hockin *et al.* (2012) and Valenzuela *et al*. (2012) reported that the gene expression levels for TCA enzymes are largely maintained during nitrogen depletion to potentially provide precursors for nitrogen assimilation, in the event more nitrogen is encountered. This explains the rapid reversal of lipid accumulation when nitrate is supplemented to the depleted cells. Metabolism is primed for assimilation of nitrogen and can quickly shift back toward growth conditions. As soon as nitrogen becomes available, carbon can be redirected from fatty acid storage to biomass growth and existing lipids are consumed (Valenzuela 2013). The obtained values of biomass productivity are in agreement with literature data (from 0.003 to 1.9 g/L/day: Mata *et al.* 2010, Rodolfi *et al.* 2009,Song, 2013).

In the bubbling column photobioreactor the highest productivity was obtained after 7 days (first harvest) reaching a value of 35.8 mg/L/day, not significantly different from biomass of the 3rd and 4th harvest that gave respectively 23 and 25 mg/L/day.The values of biomass productivity in the bubble column photobioreactor in the first three harvested were lower compared to the harvest samples growth in flask with air mixing in nitrogen starvation (Air-N).



Graphic 1: *P. tricornutum: b*iomass productivity in little volumes at 7, 14, 21 and 28 days (harv. 1, 2, 3 and 4) at different conditions: ½ SWES - Air (**Control-Air**), ½ SWES - 3% (v/v) CO_2 (**CO_2**), ½ SWES without nitrogen - Air (Air-N), ½ SWES - without nitrogen +3%(v/v) CO_2 (CO_2-N).



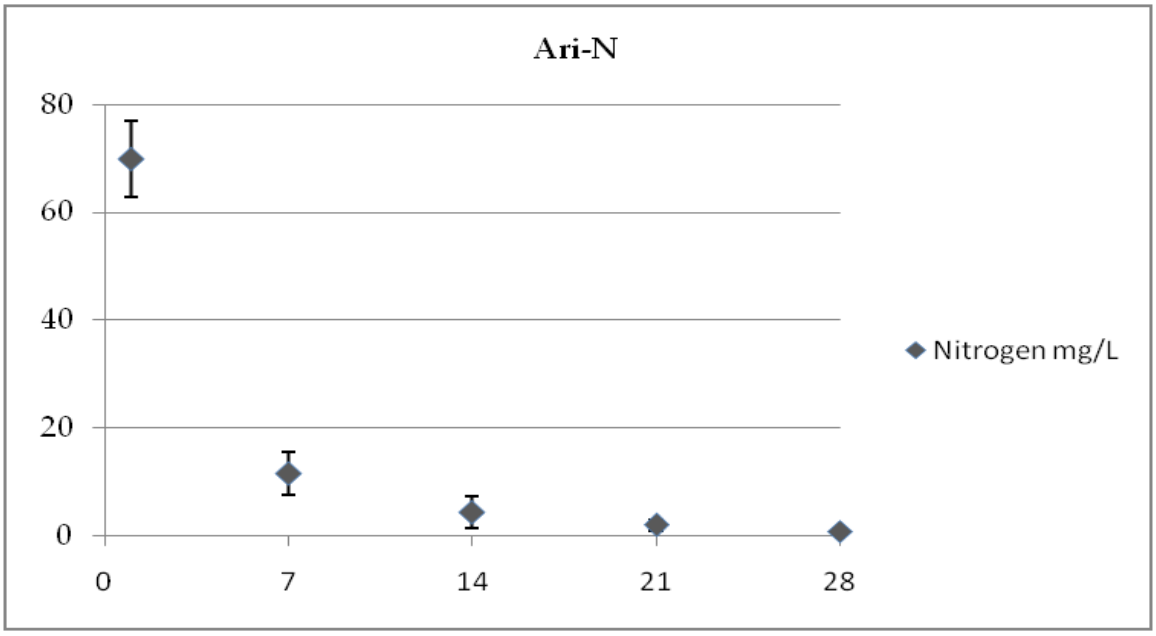
Graphic 2:*P. tricornutum:* biomass productivity in bubble column photobioreactor (250L) at 7, 14, 21 and 28 days (harv. 1, 2, 3 and 4), medium 1/2SWES.

The effects of nitrogen starvation on *P. tricornutum* lipid content (% dw) and productivity (mg/l/day) are shown in table 1. The highest values of lipid content were reached after 28 days (harvest 4) in samples grown in nitrogen starvation with air (Air -N) and with CO_2 (CO_2-N), the values are respectively of 26,9±3,5 and 26±3 % dw, significantly different respect the other treatments. The lipid percentage in the samples Air-N and CO_2-N increased when the nitrogen values were close to zero (Graphic 3 and 4), as already observed by Thomas *et al*. (1896).Zhi-Kai Yang (2013) reported that the light-harvesting complex involved in photosynthesis, including six members of fucoxanthin chlorophyll a/c proteins, showed decreased expression in *P. tricornutum* growth in medium deprived in nitrogen. Also in diatom *Cyclotella cryptica*, where in vivo

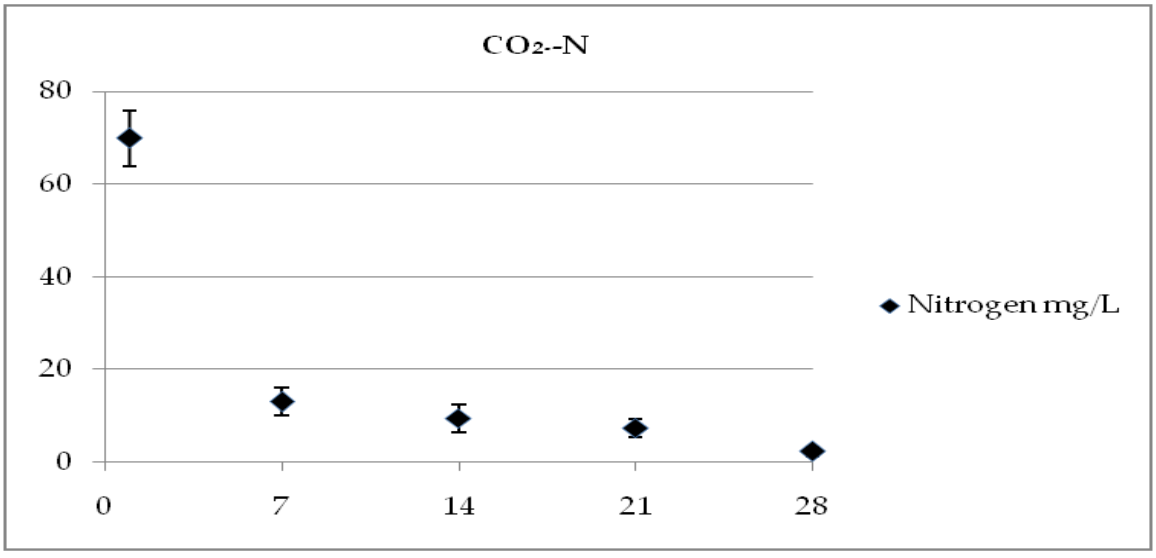
experiments using antibodies against phosphothreonine residues and in vitro studies using [γ-^{32}P] ATP showed that fucoxanthin chlorophyll a/c binding proteins of 22 kDa became phosphorylated (Brakemann *et al*. 2006). This suggests that –N(privation) could decelerate the light-harvesting process in photosynthesis. Down regulation of the light-harvesting complex under −N (privation) effectively reduces light energy absorption. Furthermore, the decrease in photosynthetic activity inhibits the relevant carbon fixation pathway. Therefore, alternative metabolic pathways may occur, which compensate for carbon assimilation and eventually lead to high lipid accumulation under –N(privation). Although the treatment in nitrogen starvation with insufflation Air gave the highest value of lipid content (% dw), the lipid productivity (mg/L/day) was the lowest respect the other samples (5.1±3 mg /L/day). In the microalgae growth in 1/2 SWES with and without nitrogen in CO_2 the highest values lipid productivity (mg/L/day) were observed in the first 3 harvested.

Table 1: Lipid productivity (mg/l/day) and content (% DW) of *P. tricornutum* harvesting at 7, 14, 21 and 28 days (harv. 1, 2, 3 and 4) at different conditions: ½ SWES - Air (Control-Air), ½ SWES - 3% CO_2 (v/v) (CO_2), ½ SWES without nitrogen - Air (Air-N), 1/2 SWES- without nitrogen + 3% CO_2 (v/v) (CO_2-N). Little volumes

	Harvest	Lipid content (% dw)	Lipid productivity (mg/ L/day)
Control	I	18,5±7	10,80±2
	II	20±5	9,56±1
	III	21±4	7,4±2
	IV	21±1,5	9,24±3
Air-N	I	22±7	13,06±2
	II	25,1±5	12,75±3*
	III	25±4,5	7.5±2
	IV	26.9±3,5*	5.1±3*
CO_2	I	16±1.5	14.24±2*
	II	19±2.3	12.35±3*
	III	21±3	10.71±1,5*
	IV	22±2	12.1±2
CO_2-N	I	17.5±4	15.81±3*
	II	20.8±5	10.77±2*
	III	24±3	12.24±3*
	IV	26±3*	10.92±3



Graphic 3



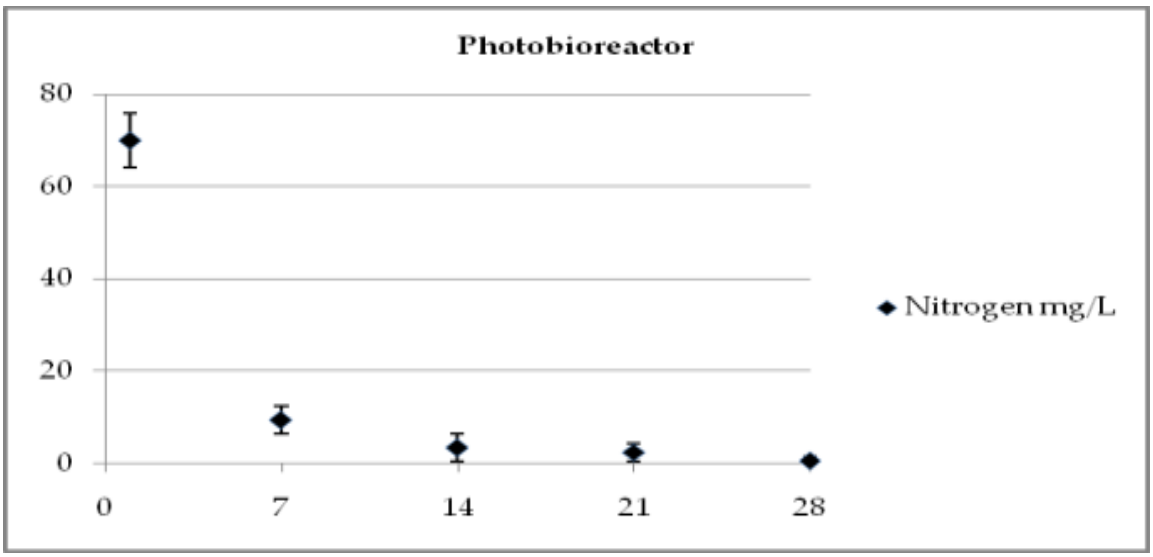
Graphic 4

Graphic 3-4: levels of nitrogen (mg/L) after each harvest in: ½ SWES without nitrogen – Air (Air-N), ½ SWES without nitrogen –CO_2 (CO_2-N). Little volumes

The lipid content (% dw) and lipid productivity (mg/L/ day) of the bubble column photobioreactor indoor, are shown in Table 2. The highest lipid content (% dw) in the photobioreactor was obtained at the 4th harvest (after 28 days) with a value of 25±2.5 mg/L/day, value not statistically different from the previous harvest (14 days). The lipid percentage increased when the nitrogen value was close to zero (Graphic 5).This values confirm what happened in the algal sample cultivated in standard medium deprived of nitrogen with air insufflation (AIR-N) in small volumes (flask 10 liter). The lipid productivity values not showed difference between harvested according to value observed in the last harvest of the sample cultivated in standard medium deprived of nitrogen with air insufflation(AIR-N) in small volumes (flask 10 liter).

Table2: Lipid productivity (mg/l/day) and content (% DW) of *P. tricornutum* harvesting at 7, 14, 21 and 28 days(harv. 1, 2, 3 and 4) with ½ SWES without nitrogen –Air. Bubble column photobioreactor indoor (250L)

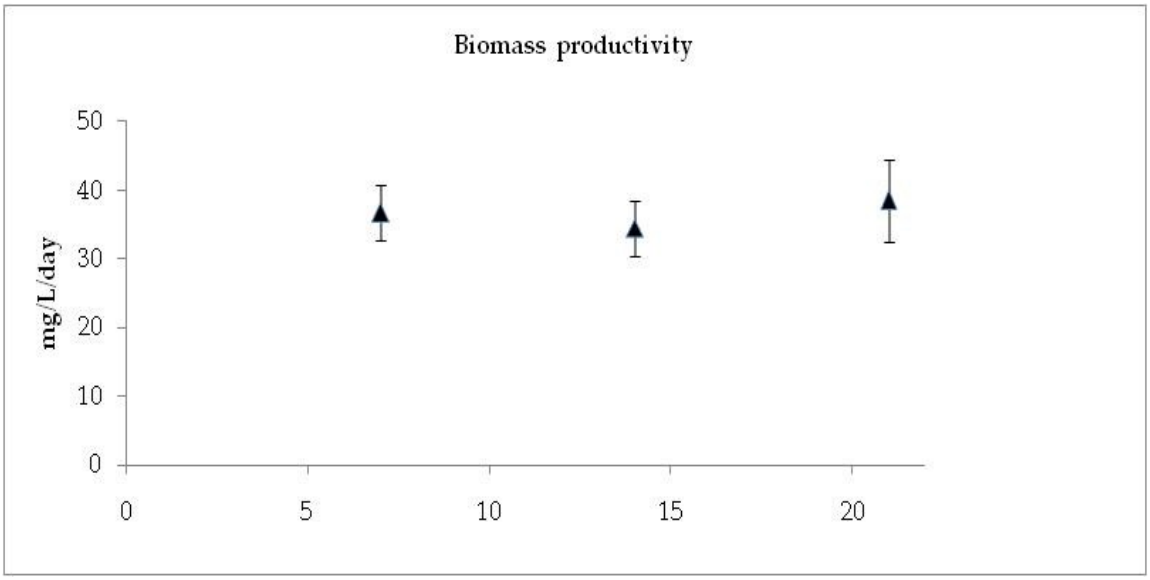
	Harvest	Lipid content (% dw)	Lipid productivity (mg/ L/day)
photobioreactor indoor	I	18±1.8[a]	6.4±3
	II	20±2[a]	6±2
	III	23±4[ab]	5.2±3
	IV	25±2.5[b]	6.25±3.4



Graphic 5: Levels of nitrogen(mg/L) after each harvest in :1/2SWES without nitrogen –Air.Bubble column photobioreactor indoor (250 L)

Cultivation outdoor (photobioreactors and ponds)

To assess the variability in biomass production and lipid content on the basis of production technologies and environmental parameters, *P. tricornutum* was cultivated under green-house in Portici (Naples) in photobioreactors (120L) and in ponds (2,5m^3) both with very low technologies and low management cost. Biomass productivity (mg/L/day), lipid content (% dry weight) and lipid amount (mg/l/day) were evaluated. In the photobioreactor the biomass productivity was 15±2 mg/L/day with a lipid content of 17.9±5.3 (% dw), while lipid productivity was 2.59±2 mg/L/day, this data were significantly lower respect to samples grown in standard medium with air in flask (Control 10L), because the small volume cultures were carried out indoor at stable condition of temperature, irradiance and photoperiod.In the large scale probably the high daytime light (see Mat and met paragraph) increased dissolved oxygen concentration that produced photo-oxidative damage to algal cells. Yusuf Chisti (2007) reported that to prevent inhibition and damage, the maximum tolerable dissolved oxygen level should not generally exceed about 400% of air saturation value.The biomass productivity (Graphic 6) considering the three harvesting was around 36±1 mg/L/day, value not comparable with the productivity obtained by Mirón *et al.* (2002) (4 kg m^3 after 12 day) but in photobioreactor in batch, with split cylinder airlift reactor, draft-tube airlift reactor . The highest lipid content (%dw) was obtained in the last harvest (13±1.58 mg/L/day), with lipid amount that was not significantly different among harvesting (Table 3). Both lipid and biomass productivity was higher than those of the photobioreactor, because the photobioreactor was cultivated in batch, while the ponds was cultivated in continuous, moreover the ponds presented a larger surface and the culture inside had an height, that did not exceed 25 centimeter allowing light penetration.



Graphic 6: Biomass productivity (mg/L/day) of *P. tricornutum* harvesting at 7, 14 and 21 days (harv. 1, 2 and 3) with ½ SWES in Air. In pond (2,5m^3)

Table 3: Lipid productivity (mg/l/day) and content (% DW) of *P. tricornutum* harvesting at 7, 14 and 21 days (harv. 1, 2 and 3) with ½ SWES in Air. In pond (2500L)

	Harvest	Lipid content (% dw)	Lipid productivity mg/ L/day
ponds outdoor	I	9±1	33±1.5
	II	10±1	3.4±2
	III	13±1.5	5.5±2.1

Fatty acid profile: cultivation indoor (little volumes and photobioreactors) - N starvation

In Table 4 the fatty acid profile (%) of *P. tricornutum* grown in small volumes in nitrogen starvation is reported. Myristc acid (14:0), Pamitoleic acid (C16:1) and Oleic acid (C18:1) did not show a statistically significant difference among samples. Palmitic acid (C16:0) was higher in some nitrogen starvation samples (Air-N, CO_2-N) and samples cultivated with 3% CO_2. Palmitic acid (C16:0) was high in some nitrogen starvation samples (Air-N, CO_2-N) and in samples cultivated with 3% CO_2, but Linolenic acid in the same samples gave significantly lower values than the control. EPA (C20:5) level was significantly higher in the microalgae cultivated in standard medium whit air (harvest 1-2-3-4 Control and harvest 1 Air-N). the other samples showed significantly lower values respect to these. Notwithstanding the EPA concentration in the sample growth in standard medium (with and without nitrogen) with CO_2 was low, it resulted better than *P.tricornutum* growth on other forms of carbon, added at values among 1.50 and 2.40% (Garcia, 2005).

Table 4: **%** Lipid Characterization of *Phaeodactylum TricornutumTricornutum* cultivation at 7 ,14 ,21 and 28 days (harv. 1 ,2,3and 4) with : 1/2SWES- Air (Control-Air), 1/2SWES- 3%(v/v)CO_2 (CO_2), 1/2SWES without nitrogen - Air (Air-N) ,1/2SWES- without nitrogen +3%(v/v) CO_2 (CO_2-N).In borosilicate flask 10L

		% Lipid Characterization					
	harvested	Myristic	Palmitic	Palmitoleic	Oleic	γ linolenic	EPA
Control	I	7±1.3	24±1.5	35±1.3	5±1.2	7±2.3	12±2.5
	II	5±1.3	25±4.5	34±6.0	6±1.5	7±1.3	15±1.2
	III	7±1.2	22±1.3	36±3.4	7±0.8	5±1.4	14±1.3
	IV	7±2.4	22±7.4	36±3.1	7±3.4	5±0.5	14±3.0
Air-N	I	7±1.21	22±1.3	36±1.5	7±1.2	5±2	14±2.5
	II	6±1	35±4*	39±6	8±2	1±1*	6±1*
	III	5±1	38±1*	37±3	7±0.8	1±1.4*	6±1*
	IV	5±2	35±7	35±3	5±3	1±1*	6±3*
CO_2	I	6±2.0	33±3.5*	37±4.0	5±1.4	2±3.8*	8±1.5*
	II	6±1.6	34±1.8*	37±44	6±1	2±2.0*	6±1.3*
	III	5.5±1.3	37±2.0*	36±2.1	6±4.0	3±2.7	7±3.1*
	IV	4±2.4	34±6.3	36±3.4	6±1	3±1	7±3*
CO_2-N	I	5.5±1	32±3	36±7	6±1	3±4	7±1*
	II	6.4±1	36±2*	40±4	7±1	1±1.5*	6±1*
	III	4.7±2	40±2*	36±2	6±4	1±3*	7±3*
	IV	4.6±2	37±5.5*	35±3	6±1	1±1*	6±3*

Table 5 reports the fatty acid profile (%) of cultures growth in the photobioreactor indoor (250L). Myristc acid (14:0) showed low value in the harvest 3, significantly different than the other harvested. Palmitic acid (C16:0) concentration increased since the second harvesting. Pamitoleic acid (C16:1)not showed a statistically significant difference among harvested. Oleic acid (C18:1)presented the highest value in the 2nd harvest (4.8±0.5 %) . γLinoleic acid (C18:3) and EPA (C20:5) showed the best results after 7 days (harvest 1).The results obtained in the first harvest for the photobioreactor at temperature and luminosity controlled were compared with harvested of small volumes (10L) grown in standard medium with air.Nitrogen content of the medium has been reported to affect the proportion of saturated to unsaturated fatty acids in many microorganisms. Under nitrogen stress, *Dunaliella bardawil* and *Dunaliella salina* produced a higher percentage of EPA (Amotz *et al.* 1985). In contrast, the proportion of polyunsaturated fatty acids in the freshwater algae *Scenedesmus* and *Chlorella* increased at high nitrogen concentrations, which is consistent with reported data, at limited nitrogen levels (Holdsworth *et al*. 1976, Thomas 1984,Yongmanitchai *et al*. 1990).Also Tonon *et al.* (2002) showed that the eicosapentaenoic acid content in *P. tricornutum* ranges from 16.1% for exponential cells to 5.8% of total lipids for stationary ones.

Table 5:% Lipid Characterization of *Phaeodactylum Tricornutum* cultivation at 7 ,14 ,21 and 28 days (harv. 1 ,2, 3 and 4) with 1/2SWES without nitrogen - Air. In photobioreactor indoor(250L)

	% Lipid Characterization						
	harvest	Myristic	Palmitic	Palmitoleic	Oleic	γ Linolenic	EPA
photobioreactor indoor	I	7.6±1[a]	26.8±3[a]	30.6±3[a]	1±0.2[a]	8.6±1.3[a]	13.9±1[a]
	II	8.7±1[a]	38.8±4[b]	26.4±3[a]	4.8±0.5[b]	4±0.6[b]	6±1[b]
	III	1±1[b]	44±5[b]	35±4[a]	2±0.2[a]	1±0.2[b]	5±0.6[b]
	IV	8±1[a]	39±3.9[b]	32±3.1[a]	2±0.3[a]	1±0.2[b]	6±0.7[b]

***Phaeodactylum Tricornutum* cultivation outdoor in photobioreactors and ponds fatty acid composition**

The table 6 showed , lipid profile(%) of P. tricornutum in photobioreactors. The fatty acids profile in this experiment is comparable for some fatty acid with air control (Control-Air) of first experiment. The fatty acid with the highest percentage was oleic acid 14%. The decrease in the unsaturation level of fatty acids as shown in this case is well known during the temperature shift in a wide range of living cells (Sato N, Murata N 1981)

Table 6: **%** Lipid Characterization of *Phaeodactylum Tricornutum* cultivation at 14 days in 1/2SWES with- Air. In photobioreactor outdoor (100L)

	%Lipid characterisation					
	Myristic	Palmitic	Palmitoleic	Oleic	γ Linolenic	EPA
photobioreactor outdoor	6±2.1	26±4.3	23±4	14±4.5	8±2	10±1

The fatty acids percentage in *P.tricornutum* cultivation in ponds are shown in Table 7. Myristic acid (14:0), Oleic acid (C18:1) and γ-Linolenic did not a statistically significant difference among harvested. Palmitic acid (C16:0) in 2° and 3° harvested presented values significantly different compared to the first one. High concentration of EPA were observed in the first harvest (25% of total fatty acids), also found in the literature (Fajardo et al.l 2007,Mirón et al.l 2003) and greater than that contained in the oil cod liver, that has about 18%. Renaud *et al* 1995 reported that in *Isochrysis galbana* increased in EPA content at a temperature of 20°C.

Table 7:% Lipid Characterization of *Phaeodactylum Tricornutum* harvesting at 7, 14 and 21 days in ½ SWES with- Air. In photobioreactor outdoor in continus (1600L)

	% Lipid Characterization						
	harvest	Myristico	Palmitic	Palmitoleic	Oleic	γ-Linolenic	EPA
ponds	I	6±2.1	18±2	19.3±1.8	13.9±1	3.1±0.9	25.7±3
	II	4±3	30.7±3.6*	27.4±3*	13.4±1.5	4.8±1	17.6±2*
	III	4±2	29.1±3*	25.4±2.5*	12.3±1.5	4.2±0.6	15.3±1.5*

4.3 Conclusions

This study showed starvation nitrogen showed to be a good treatment to increase lipid percentage. CO_2 was a good treatment to increase biomass productivity, that lipids content and productivity of *P.tricornutum* was significantly enhanced using treatments as nitrogen starvation with and without CO_2,but the best technology production was the pond due to EPA concentration and low cost management and maintenance. It is estimated that the pond has a cost 10 times lower per liter of biomass produced compared to a photobioreactor at PMMA luminosity and temperature controlled with a bubble column air. A further optimization of productivity, and reduction of production costs, which are realistic in a 5 year perspective see the production of EPA and DHA of microalgae has the potential to develop into a sustainable alternative to fish oil for use in aqua-feed. This potential can be realized by establishing a fit-for-purpose research and development pipeline with integrated research along the value chain in light of the recent price development and the future fish oil

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Chapter 5

Lipid content and fatty acid profile of *A. maxima* cultivated in south of Italy

Introduction

The genus *Arthrospira* belongs to the group of systematic Cyanobacteria, ancient prokaryotic organisms appeared on Earth three billion years ago. *Arthrospira* (Spirulina) is an edible cyanobacterium that performs prokaryotic oxygen-evolving photosynthesis, .converting CO_2 into organic compounds using the energy from sunlight (Ciferri 1983, Vonshak 1997). It often dominates the plankton of warm lakes that have high carbonate/bicarbonate content and pH levels (Grima *et.al* l999, Ciferri 1983, Vonshak1997). The species marketed in food industries with the name of "*Spirulina*" are *Arthrospir*a maxima and *Arthrospira platensis* (Cohen *et al.* 1995, *Viti et al.* 1997).
The cyanobacterium Spirulina has been used as a food for centuries by native peoples from Lake Chad in Africa and Lake Texcoco in Mexico (Vonshak 1997, Henrikson 1994), at the moment it is used as food supplement for undernourished people in many parts of the world (Henrikson, 1994) due to its high level of various pigments (e.g., chlorophyll a, phycocyanin and carotenoids), proteins (about 60-70%of dry weight), vitamins, minerals, and some essential fattyacids (e.g., γ-linolenic and eicosapentaenoic acids) (Apt *et al.* 1999, Choi et al. 2008, Ciferri 1983, Franke *et al.*1994, Sanghvi *et al.* 2010, Vonshak1997) Preparations of *Spirulina*, sold in capsule form or in foods such as beverages, pastes, biscuits have been shown to have therapeutic properties in the treatment of conditions such as hypercholesterolemia and atherosclerosis (Ramamoorthy and Premakumari, 1996), pre-menstrual tension and arthritis and as an auxiliary in weight loss (Henrikson, 1994). Among the compounds present in Spirulina biomass, gamma-linolenic acid γ-Linolenic (C18:3, ω6,) and phycocyanin are received most attention from researchers. Phycocyanin was first studied as food colorant (Sarada *et al*. 1999), while γ-Linolenic was mainly studied for its therapeutic and nutraceutical properties such as its ability to decrease blood cholesterol levels (Ishikawa *et al.* 1989). There are a lot of researches on Spirulina as a potential source of γLinolenic (Alonso and Maroto 2000, Quoc *et al.* 1994, Cohen *et al.* 1987, 1993) and the growth conditions showed to influence the γ-Linolenic content. *Spirulina* is cultivated mainly in tropical and subtropical regions, but also where temperate zones the climatic conditions can induce a high biomass productivity and profits. Several studies evaluated the Spirulina productivity in the north countries of the Mediterranean such as Spain (Jimènez *et al*. 2002) and Italy (Zittelli *et al*. 1996) but there are no consistent data about changes in the composition of the biomass during a production season. Some researchersconsidered the variation of the chemical composition of Spirulina in response to different climatic regimes or light intensity variation, but all experiments were conducted in the laboratory-scale, using cultures in flasks or in photobioreactors.The main purpose of this work is to evaluate the biomass productivity, lipid content and fatty acid profile of *Arthrospira maxima* cultivated in pond under greenhouse in South of Italy (Portici , Naples)from June to November in two years - 2012 and 2013.

5.1 Materials and methods

Strain and cultivation

The species *Arthrospira maxima*(SAG 84.79), obtained from University of Goettingen, was selected for this experiment and cultivatedat University of Naples *Federico II*(Criacq).The strain was grown and maintained routinely in Zarrouk medium, in standard condition (temperature of 26 ± 2 C°, under photonic flux of 174 μE/m²/s - fluorescent lamp, photoperiod of 24 h light). The inocula were axenically prepared.

Cultivation in pond under greenhouse

Arthrospira maxima was cultivated in 3 ponds of 11m^2 (final volume 2,5 m^3) coated in PVC, suitable for foodproduction in Zarrouk medium. The experiment was carried out from June to November for two years (2012-2013), under constant aeration. The data of environmental conditions as Light, photoperiod and temperature were provided by ENEA in Portici (NA) and by Ministry of Agriculture Forestry and Environmental.

The culture harvest was carried out, at exponential growth phase, from two to five times a week, using a pre-filter of 400 μm and a filter of 50 μm. Between 2012 and 2013, a remarkable improvement in harvesting performance was made using a harvesting system more efficient and semi-automatic,with higher energy consumption.One time a week, based on the dried biomass harvested a replenishment of nutrient salts were made.

Analytical methods

Microalgae growth was directly monitored by optical density (OD 560nm), pH and temperature (°C). The organic dry weight was estimated gravimetrically (APHA, Standard Methods, WPCF 1995).Several biomass rates were freeze and lyophilization for subsequent analysis.was made

Lipid productivity and lipid profile

Algal biomass *Arthrospira maxima* was tested for total lipids (Folch *et al.* 1957) as percentage of dry weight. The fatty acids profile was performed by gas chromatography, after derivatization to fatty acid methyl ester (FAME) with 2 N of KOH in methanol, according to the IUPAC standard method FAME, including methyl octanoate, were analyzed on a Perkin Elmer AutoSystem XL gas chromatograph (Perkin Elmer, Waltham, MA, USA) equipped with a PTV(programmed temperature vaporizer), a flame ionization detector, and a capillary column 100 m -0.25 mm inner diameter, film thickness of 0.20 mm. Stationary phase 50% cyanopropyl methyl silicone (Supelco, USA).

The carrier gas, helium, was introduced at a flow rate of 20 cm/s. The oven temperature program was as follows: 120°C for 5 min, 5°C/min ramp to 165°C for 5 min, and then 10°C/min ramp to 240°C for 20 min. The split ratio was 1/60 and the flam ionization detector temperature was set at 260°C (Romano *et al.*2013). Peaks identification was obtained using the external standard Supelco TM 37 FAME mix (Supelco) by comparing the retention times with the pure standard components.

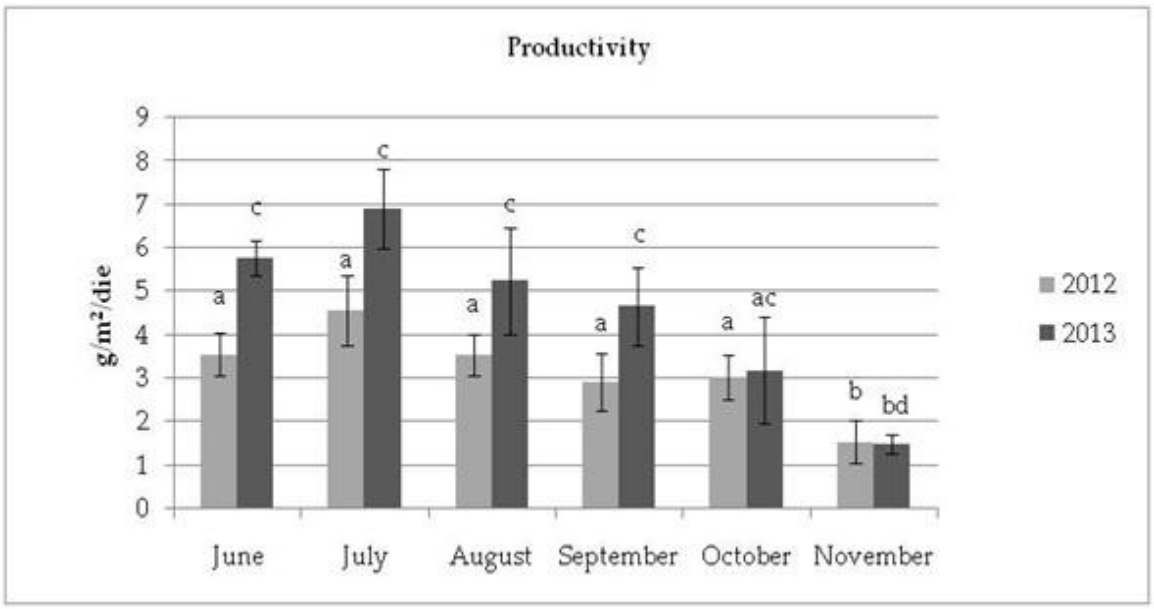
Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA). A value of $P< 0.05$ wasconsidered significant. Each value was based on three repetitions, and the corresponding data are presented as mean ± standard deviation(n = 3).

5.2 Results and discussion

Biomass productivity

The biomass productivity of Spirulina was reported in graphic 1 (production season from June to November for two years: 2012-2013).The biomass productivity (g/m^2/day) in 2013 was significantly higher respect to 2012 production,from June to September,but was not different in October and November.



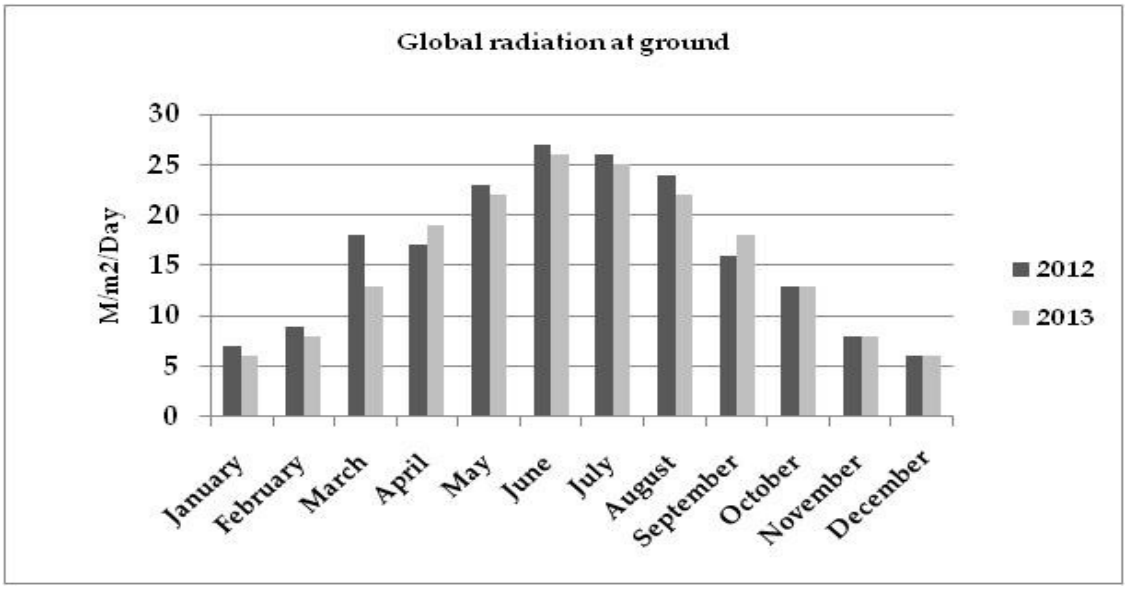
Graphic1: Biomass productivity (g/m^2/ day)of Spirulina years 2012 2013.

The average productivity of the summer 2012 was 3.88 g /m^2/day, lower than value obtained the second summer: 6 g/m^2/day. The ponds in 2013 in fact, had a total production of 37.4% higher than the previous year. This difference is not related to the climatic conditions as temperature and radiation (see Graphic 2) were not significantly different in the two years, but to the improvement of the harvesting and culture mixing systems.

In 2012 the culture mixing was provided through the air bubbling, while in 2013 a wheel was applied, moreover the harvesting efficiency in 2013 was increased through the use of a semi-automatic system, that allowed to collect a triple quantity of the biomass in the unit of time, compared to the rudimentary system used in 2012.

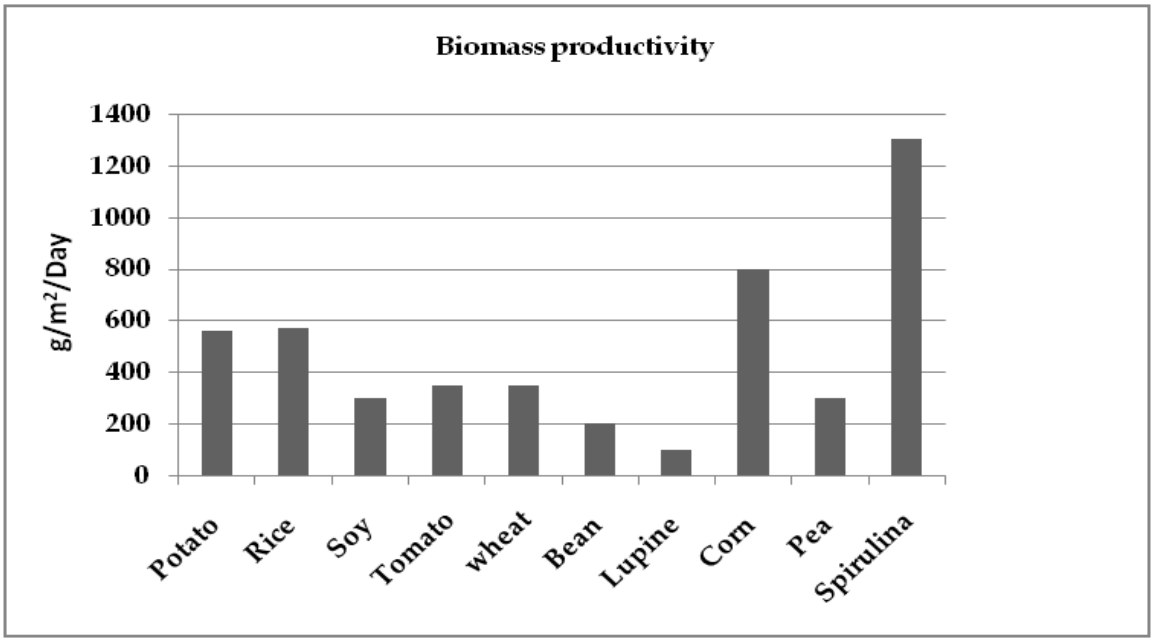
During the months of June, July and August, the productivity was 43% higher than that obtained in the following period (September, October, November). This reduction in productivity is absolutely a normal and expected behavior, as reported also in the work of Zittelli *et al.* (1996), mainly induced to the radiation and temperature reduction in autumn and winter respect to the summer.In this work, the winter biomass productivity decrease compared to the summer months was in the order of 20% in the autumn to get to 60% in the winter. Even in the work of Richmond et al. (1990) was reported a similar decline in productivity, which increased to 25-27 gm^{-2}d^{-1} for the period from May to October, starting to 12-14 gm^{-2}d^{-1} for the next 6 months (November to April).

The productivity data of the present work were low if compared to the studies reported in literature, but a simple comparison is not exhaustive, considering that the production was made applying low technologies with minimum costs. Jiménez *et al*.(2003) reported a*Spirulina*production in southern Spain in 9 months (except winter) with an average potential productivity of 10.3 gm^{-2}d^{-1} (DW), which decresead to 8-2 gm^{-2}d^{-1}(DW)over 12 months.



Graphic2: Daily global irradiation performance in Naples from 2012-2013.

Graph 3 shows the annual productivity (g/m2/y) of *Spirulina* in 2013 compared with the productivity of some common agricultural products (cultivated in Italy from 2008 to 2012) (FAO official data - STAT , 2013). The high Spirulina productivity stands out, showing a value 40% higher respect to corn and more than double respect to rice and potatoes (in terms of dry weight).



Graphic 3 : Comparison of Spirulina productivity with several agricultural products in Italy

Fatty acid and lipid profile :

InTable 1 the lipid content (%dw) and productivity (g/m²/day) of *A. maxima* in 2012 and 2013 years were reported.In the year 2012 the months June and July showed values of lipid content(%dw)significantly lower that other months.In the 2013 the lipid content was not significantly different among months, and the values obtained in the months August,September and November were not significantly different respect 2012 harvested.The mean percentages between the two seasons of production were, respectively 8.0±3.2 in 2012 and 8.3 ± 2.2 in 2013.

Table1: Content(%dw) and productivity Lipidi (g/m²/day) of Arthrospiara maxima in 2012 and 2013 years

	2012		2013	
	Lipid content (%dw)	Lipid productivity (g/m²/day)	Lipid content (%dw)	Lipid productivity (g/m²/day)
June	4±1a	0,14±0,02a	7±0,2d	0,35±0,04e
July	5±1a	0,22±0,02b	9±1d	0,62±0,05f
August	7±2b	0,21±0,02b	9±2db	0,49±0,04g
September	11±1c	0,24±0,03b	12±2dc	0,52±0,04g
October	12±2c	0,36±0,04c	6±2d	0,19±0,03h
November	9±4c	0,14±0,01a	7±3dc	0,11±0,04ha

The data of Bujar *et al.* (1970), Challem *et al*. (1981), Earthrise Spirulina Farms (1986) and Santillan (1974) indicated a lipid content variable between 6-7% (DW), values slightly lower than mean values found in the present work.The highest lipid productivity in 2012 was registered in October, while in 2013 the highest value was in July, values significantly different respect other crops.Focusing the discussion on a healthy point of view, it is important to consider that the sum of two polyunsaturated fatty acids , linoleic acid and γ–linolenic, was about 50% of total fatty acid (Table 2). In 2012 the linoleic acid showed little variability, with a content ranged 15% to 21% and the γ -linolenic acid showed a fluctuating behaviour, with an average value of 27% - minimum value in September (14%) and maximum in June (39%). In 2013 the percentage of linoleic acid was lower with high variability and also for the γ -linolenic acid the situation was similar.
Some authors reported significant variations in polyunsaturated content related to the culture temperature changes. Mühling *et al.* (2005) showed that at 20° C - culture temperature – the values of palmitic acid was lower than 30° C, but the content of linoleic acid and γ –linolenic was higher. In the present study it was not detected a clear correlation between temperature and fatty acid profile. Lang *et al.* (2011) studying the same our strain of Spirulina (SAG 84.79) found a percentage of γ-linolenic acid on total lipid not different by the results obtained in this research (24.8%), also showing a high variability in the other species.

Table 2: Linoleic and γ-Linolenic content (%) of *Arthrospira maxima* cultivated for two years (2012-2013) in south of Italy

Month	Year 2012		Year 2013	
	Linoleico	γLinolenico	Linoleic	γLinolenic
June	21±2[a]	39±2[a]	18±2[ba]	28±2.4[d]
July	19±3[a]	18±3[b]	18±2.5[ba]	37±3[e]
August	15±4[a]	31±2.5[c]	15±3[ba]	29±2.8[d]
September	20±1[a]	14±1.7[b]	13±3[b]	36±2[e]
October	21±1[a]	27±2.1[c]	10±3[c]	34±2.1[e]
November	19±4[a]	34±3[a]	18±2.4[ba]	31±2.7[d]

5.3 Conclusions

Actually, commercial scale production facilities of *Spirulina* are mainly allocated in sub-tropical regions.The improvement of *Spirulina* growth conditions is usually very important to start a commercial production, that can be economically and nutritionally interesting. In literature there are many scientific works on the *Spirulina* cultivation but a lot of them were carried out indoor in small volumes, using photobioreactors, that although providing excellent performance of growing, do not represent a viable and economic sustainable way to massive microalgae production. The researches carried out on large volumes (outdoor ponds or raceways), at our latitudes, reported very high productivity (near 30 ton/ha/year) but often using expensive equipments with also high management costs.It is important to underline that in literature there is a lack of information about the possible variations in *Spirulina* biomass composition related to the seasonal climatic changes, especially at our latitudes. The present work showed the possible influence of environmental factors on the biomass and lipid productivity of *Spirulina*, although the lipid profile did not show significatively changes trough the years.That the light radiation and the temperature, while having a positive influence on the productivity, do not seem to have the same effect as regards the lipid composition. The total lipid content and its composition did not show a trend correlated with meteorological parameters. A.maxima literature data claim biomass productivity at our latitudes of about 30 ton/ha/y (Jimenez *et al.* 2003) so with 8% lipid productivity could reach 2,4 ton/ha/y of oil. The results of this work will be useful for the development of commercial cultivation of an innovative product in Campania, providing data on both the productive potential, both on the variability of the product during the seasons in terms of fatty acid profile.

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6. Final Conclusions

Consumers around the world are moving toward functional foods as a way to preserve and improve their well being. They are beginning to search for not only low-fat and low-sugar products, but also for foods considered as natural or with ingredients taken from natural sources as opposed to synthetically produced ingredients. Consumers are searching for food products that will help them prevent and fight diseases, increase their energy and wellness, and help them live longer, healthier, and productive lives. With that in mind, food scientists can find in microalgae a novel source for wholesome food and bioactive ingredients. Microalgae have been around "forever," but only in the last few decades have been produced and marketed as nutraceuticals and food supplements. Their potential is so much greater than the current applications. Genera such as *Spirulina, Chlorella, Dunaliella*, *Haematococcus, Schizochytrium*, and *Isochrysis* have become popular microalgae sources of protein-rich biomass and compounds, especially carotenoids, pigments, antioxidant extracts, and essential fatty acids.The acceptance of the use of microalgae biomass or biomolecules extracted from it has resulted in the development of various innovative food products enriched with microalgae or their sub-products. Up to the moment, very few such products have appeared in the health and natural sector shelves of stores across Europe and Asia, therefore, there is still a very large untapped opportunity in this food area. This study aiming to improve in different way lipid profile and relative production of microalgae *B.braunii*, *P.tricornutum* and cyanobacteria *A.maxima*, has focused attention on some fatty acids of food interest (Table 1).

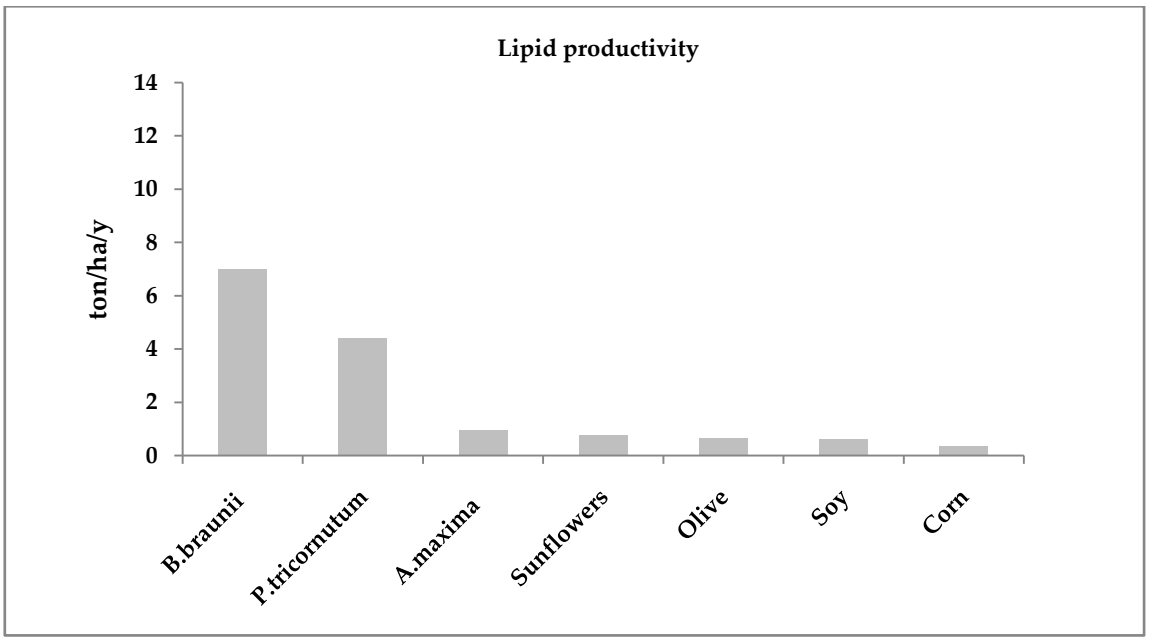
Table 1: Algal fatty acids considered in this study

Fatty Acidi	Possible health effect
Palmitoleic acid	reduce risk of certain heart diseases
Palmitic acid	antimicrobial activity
Oleic acid	antioxidant activity
Linoleic acid	lowering of cholesterol
γ– Linolenic acid	anti-inflammatory and immune-modulating activity
EPA fatty acid	reduce risk of certain heart diseases

B.braunii SAG: Showed that the lipid productivity and content was significantly enhanced using treatments as nitrogen starvation with and without CO_2, and using sodium bicarbonate as C source.The fatty acid profile of *B. braunii* resulted very interesting for application in food and feed industries, being characterized by some very important fatty acid as Palmitic, Stearic, Oleic, Linoleic and γ-Linolenic, which composition and quantity can be modulated applying different culture conditions. Comparing the obtained lipid profile of *B. braunii* with some vegetable oils was observed that , linoleic acid amount is sometime comparable vegetable if oils used in human food and for animal feed, while γ-Linolenic acid percentage shows values higher than level reported for vegetable oils. Due to the high presence of γ-Linolenic acid, important for its anti-inflammatory and immune-modulating activity, and due to the presence of polyunsaturated fatty acid, a lot of application in human food and animal feed could be developed. *B.braunii* U-TEX

and IBL strains have shown to be a good source of biodiesel for their ratio between fatty saturated and unsaturated.

Phaeodacctylum tricornutum: lipids content and lipids productivity were significantly enhanced using treatments as nitrogen starvation with and without CO_2, and the best production technology, due to EPA concentration and low cost management and maintenance was the pond. The fatty acid profile of *P. tricornutum* resulted very interesting for application in food and feed industries. For the presence of EPA that is very important for his function in the anti-inflammatory activity, anti-cancer and for the treatment of developmental disorders and mental health. *A.maxima*: Albeit light radiation and temperature have a positive influence on the productivity do not seem to have the same effect on lipid composition. The total lipid content and its composition did not show a trend correlated with meteorological parameters. The results shown the possibility, at our latitudes, to produce *Spirulina* under greenhouse with excellent nutritional value for most of the year and with good production rates maintaining low operating costs (energy and management) at low level. This results could be useful for the development of commercial cultivation of an innovative product in Campania, providing data on both the productive potential and on the variability of the product during the production season. In some European countries n-3 FA daily intake in adults vary between 80 and 420 mg/d. EU recommended daily intake of Omega-3 EPA/DHA is 250 mg/d. In order to achieve a "Source" claim a min. 15% RDI (i.e. 37.5 mg EPA+DHA) in 100g or 100ml product will be needed and to achieve a "Rich Source" claim a min. 30% RDI (i.e.75mg EPA+DHA). EFSA proposes, for n-6 PUFA, 10g as labelling reference intake value, which is consistent with recommended intakes for adult individuals in the population. In accordance with the EFSA statements, taking into consideration the algae used in this work, 20 gr of *B. braunii* oil and 24 gr of *A.maxima* oil could met the daily intake of n-6. The assumption of 1 gr of *P. Tricornutum* oil would be satisfied the daily ration of EPA.



Graphic 1: Lipid productivity of microalgae *B.braunii, P.tricornutum, A.maxima* cultivated with low technology in mediterranean latitude and lipid productivity of vegetable commonly used as a source of food oils.

It's important to underline that microalgae as *B. braunii* and *P. tricornutum*, cultivated in south of Italy, with low-cost technologies (in a period of 200 days) showed lipid productivity higher if compared to the lipid productivity of Sunflower, Olive, Soy and Corn (data obtained from http://faostat3.fao.org and http://www.inran.it)(Graphic 1).*A. maxima* showed a lipid productivity comparable to the sunflower, although the main food characteristics of *A. maxima* is the protein content, that can reach the 60% of the dried weight (Beckeret al. 2007). The estimated lipid productivity of 4.4 ton/ha/y for *P.tricornutum*is in the range (from 0.86 to 23 ton/ha/y) reported by Mata *et al*. (2010).Some authors for *B.braunii* reported a lipid productivity ranging from 1.5 to 4.5 ton/ha/y, value 30% lower than our results in agreement with values reported by Asnokkomor et al. (2012) whit a lipid productivity of 6.5 ton/ha/y. Literature data on *A.maxima* claim biomass productivity at our latitudes of about 30 ton/ha/y (Jimenez *et al*. 2003)so with 8% lipid productivity could reach 2.4 t/ha/y of oil. Commercial scale facilities of *Spirulina* produce between 10 to 30 ton/ha/y, so lipid productivity should be between 0.8 and 2.4 ton/h/y (Sasson 1997) our data of 0.96 using low-tech cultivated process in agreement with these data. In conclusion, considering the results obtained in this study and the literature data that indicate microalgae fatty acids as a panacea for human heath (e.g. antioxidant, immune-stimulating, antimicrobial) with also some possible use as a drug delivers (as an example *B. braunii* oil may be useful enhancers for flurbiprofen delivery via the skin as reported by Fang 2004), we can consider *A.maxima, P.tricornutum* and *B.braunii* as a good source of oil to be used as a functional ingredient in food industry or for the market of food supplements. These microalgae could be cultivated at our latitudes at relatively low cost of production with good nutritional characteristic through the year.

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Electronic materials

http://www.inran.it

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Appendix 2 List of publications and participation in conferences:

Publications

1. Silvia B, Langellotti A.L, Martello A., Francesca R., Fogliano V. 2014. Functional Ingredients from Microalgae (Submitted).

2. Fabbrocini A., D'Adamo R., Del Prete F., Langellotti A.L., Barone C.M.A., Rinna F., Sessa R., Silvestri F., VillaniG., Vitiello V., Sansone. 2013. Motility of cryopreserved spermatozoa for the ecotoxicological evaluation of aquatic environments. Chemistry & Ecology 29 (7): 660-667. ISSN:1027-2860.

3. D'Adamo R, Del Prete F, Langellotti A.L, Rinna F, Silvestri F, Sorrenti G, Vitiello V, Sansone G, 2012.Cryopreserved semen in ecotoxicological bioassays: sensitivity and reliability of cryopreserved Sparus aurata spermatozoa. Ecotoxicology and Environmental Stafy 84:293-298 ISSN: 0147-6513.

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Comunication at congress

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The sperm motility in marine teleosts as a tool to evaluate the toxic effects of xenobiotics

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The possibility of using the sperm of teleosts as a model system for ecotoxicological assessments has been explored by evaluating sperm motility parameters: (1) time to reach the maximum motility value (activation time), (2) maximum motility value, (3) duration of maximum motility value, and (4) total time of motility (until class 0). Sperm of *Dicentrarchus labrax*, *Sparus aurata*, *Diplodus puntazzo* and *Pagellus erythrinus* were analysed and compared. The effects of dimethylsulfoxide, ethylene glycol, propylene glycol, glycerol and methanol on sperm motility in these marine species were investigated. Among the systems tested, sperms of *S. aurata* and *D. labrax* were the most sensitive to the tested xenobiotics and *S. aurata* spermatozoa were shown to be easier to manage for ecotoxicological assays.

Keywords: marine teleosts; sperm motility; xenobiotics; ecotoxicological assays

1. Introduction

Toxicity tests to assess ecosystem contamination are now used alongside, if not to replace, analytical methods that directly search for pollutants, because toxicity tests provide more accurate information about the actual effect of the toxic on natural ecosystems, even at low concentrations. Factors that must be taken into account when determining the value of a biological system as an indicator in toxicity tests are the sensitivity of the system to different pollutants, and the year-round availability of the organism and its gametes and embryos.

Because of their high sensitivity, the gametes and embryos of aquatic organisms are commonly used in ecotoxicological tests to assess the quality of waters and sediments in areas subject to anthropogenic effects [1–7].

Reproductive capacity is, in fact, a key factor in the survival of a species, therefore, these biological systems can serve as valuable tools in assessing the environmental risk posed by chemical contamination.

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Cryopreserved semen in ecotoxicological bioassays: Sensitivity and reliability of cryopreserved *Sparus aurata* spermatozoa

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ABSTRACT

The aim of this study was to evaluate the feasibility of using cryopreserved *S. aurata* semen in spermiotoxicity tests. Cryopreservation is a biotechnology that can provide viable gametes and embryos on demand, rather than only in the spawning season, thus overcoming a limitation that has hindered the use of some species in ecotoxicological bioassays.

Firstly, the sperm motility pattern of cryopreserved semen was evaluated after thawing by means of both visual and computer-assisted analyses. Motility parameters in the cryopreserved semen did not change significantly in the first hour after thawing, meaning that they were maintained for long enough to enable their use in spermiotoxicity tests. In the second phase of the research, bioassays were performed, using cadmium as the reference toxicant, in order to evaluate the sensitivity of cryopreserved *S. aurata* semen to ecotoxicological contamination.

The sensitivity of the sperm motility parameters used as endpoints (motility percentages and velocities) proved to be comparable to what has been recorded for the fresh semen of other aquatic species (LOECs from 0.02 to 0.03 mg L^{-1}). The test showed good reliability and was found to be rapid and easy to perform, requiring only a small volume of the sample. Moreover, cryopreserved semen is easy to store and transfer and makes it possible to perform bioassays in different sites or at different times with the same batch of semen.

The proposed bioassay is therefore a promising starting point for the development of toxicity tests that are increasingly tailored to the needs of ecotoxicology and environmental quality evaluation strategies.

1. Introduction

Current strategies in monitoring programs for marine-coastal areas usually require the integration of chemical analyses and biological testing in order to better evaluate the bioavailable fraction of toxicant actually interacting with living organisms (Macova et al., 2010; Coulaud et al., 2011). In terms of biological parameters, reproductive success being a crucial factor in determining the survival of a species, ecotoxicological bioassays using gametes and embryos of aquatic species are widely employed (Losso et al., 2007; Mamindy-Pajany et al., 2010). Toxicants elicit a variety of effects depending on the species and the different synergistic and/or antagonistic effects of bioavailable substances present in combination. Therefore test batteries need to include organisms representing different phyla and different trophic levels (Macken et al., 2009). Organisms used in ecotoxicological bioassays should also be ecologically relevant and easily available all year round, and they should have well-known and above all homogeneous physiological responses. Moreover, they should come from the monitored area, or at least from similar environments, so that it is not necessary to alter the chemical and physical characteristics of the tested matrices and therefore the reactivity of the toxicants present. In addition, the relative endpoints should be selected so that they can be accurately, predictably and reliably measured (Chapman, 2002).

Although ecotoxicological tests have been standardised for gametes and embryos from a range of aquatic species, in many cases their availability (limited to the spawning season), the difficulty in collecting and storing them until the tests are carried out and above all their potentially high biological variability still limit their use (Paredes and Bellas, 2009; Schipper et al., 2008).

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The sperm motility in marine teleosts as a tool to evaluate the toxic effects of xenobiotics

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Motility of cryopreserved spermatozoa for the ecotoxicological evaluation of aquatic environments

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A new approach to environmental studies was investigated by the authors, who propose the use of cryopreserved biological systems in ecotoxicological bioassays. The feasibility of spermiotoxicity tests using cryopreserved semen of the sea bream *Sparus aurata*, with sperm motility parameters as the endpoint, was evaluated. Thawed sperm was incubated in environmental samples (sediment elutriate and dumpsite leachate) and in a reference toxicant (cadmium) at scaled concentrations. Motility was then evaluated by video-microscopy using both visual and computer-assisted analyses. Activation time, sperm motility and velocity and motility duration were assessed on thawing and at the end of the incubation time, and the difference with respect to the control was statistically evaluated. All the endpoints of the bioassay proved to have good sensitivity even at the highest dilutions of the tested matrices. Observed differences in the sensitivity thresholds of the endpoints were considered to be representative of different aspects of sperm physiology. Therefore the proposed bioassay is a promising starting point for the development of toxicity tests that are increasingly tailored to the needs of ecotoxicology and environmental quality evaluation strategies for aquatic environments.

Keywords: ecotoxicological test; sperm motility; computerised sperm motility analyses; cryopreservation; sea bream; dumpsite leachate; sediment elutriate; cadmium

1. Introduction

The increasing worldwide interest of policy-makers in the evaluation of aquatic ecosystem quality and in pollution prevention and control has given rise to a need for toxicity assessment tools that are suited to the specific environment being monitored.[1] Ecotoxicological bioassays using the gametes and embryos of aquatic species are commonly used in integrated environmental monitoring programmes due to their high sensitivity to the synergistic and/or antagonistic effects of all components interacting with the biota.[2] However, the use of gametes and embryos in ecotoxicological tests is limited by many factors: their availability (often limited to the spawning season), the difficulty in collecting, transferring and storing them for long periods, and above all their biological variability.[3,4] Cryopreservation is a biotechnology that provides viable gametes

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