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MICROALGAE PRODUCTION IMPROVEMENT FOR THE ICCM`S MARINE FISH HATCHERY

MAURICIO MORENO ALVA

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Organizado conjuntamente por la Universidad de Las Palmas de Gran Canaria (ULPGC), el Instituto Canario de Ciencias Marinas (Gobierno de Canarias) y el Centro Internacional de Altos Estudios Agronómicos Mediterráneos (CIHEAM), a través del Instituto Agronómico Mediterráneo de Zaragoza (IAMZ)

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Trabajo realizado en los laboratorios del Instituto Canario de Ciencias Marinas (ICCM) e Instituto Universitario de Sanidad Animal y Seguridad Alimentaria (IUSA) de la Universidad de Las Palmas de Gran Canaria, España, bajo la dirección de la Dra. Carmen M^{a}. Hernández Cruz (Universidad de Las Palmas de Gran Canaria, España) y del Dr. Francisco Javier Roo Filgueira. (Instituto Canario de Ciencias Marinas).

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Directores:

_____ _____

Dra. Carmen M^{a}. Hernández Cruz Dr. Francisco Javier Roo Filgueira

Autor:

_____

Mauricio Moreno Alva

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“Science never solves a problem without creating ten more”

Shaw, Bernard

A mi familia, a México...

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ABSTRACT

In order to improve the microalgae production in the ICCM`s marine fish hatchery one experiment took place in the traditional culture bags inside the microalgae room adding CO_2 to avoid carbon limitation, stabilize pH and boost the cell concentration; after 10 days of culture non differences were observed. Microalgae growth could be limited for other factors, such as nutrient and light limitation, so a second experiment took place this time adding twice the regular nutrient dose plus CO_2 gas income to the culture bags for 20 days. After the 10^{th} culture day different dilutions (10%, 15% and 20%) were tested to obtain the best dilution rate for a daily microalgae harvest.

The best dilution rate was 10% for bags without CO_2 and 15% with CO_2 for a daily harvest, although cell concentration growth didn`t show any considerable increase which lead to test outdoor photo-bioreactors to improve the cell concentration growth. Two outdoor vertical photo-bioreactors were tested and confronted to a traditional culture bag with twice nutrient dose; both photo-bioreactors (with twice and fourth times nutrient dose) were also confronted to each other. After the 10^{th} culture day, 10% of the volume was daily harvest and replaced with the same volume of enriched water on the three systems. The cell concentration showed significant magnitude increase ($P>0.05$) in the photo-bioreactors in respect to the culture bag, and also between them but not in magnitude terms.

In the last experiment microalgae growth was tested for 30 days with twice nutrient dose in a tubular horizontal photo-bioreactor system whith controlled temperature and pH by adding CO_2 gas into the culture medium. After the 10^{th} culture day, 10% of the volume was daily harvest and replaced with the same volume of enriched water. Cell density increased before it collapsed due to diatoms stuck to the methacrylate tubes.

1. Introduction

1. Introduction

Capture fisheries and aquaculture supplied the world with about 110 million tons of food fish in 2006, providing an apparent per capita supply of 16.7 kg (live weight equivalent), which is among the highest on record in the world history of food production reported in SOFIA 2008 (Figure 1).

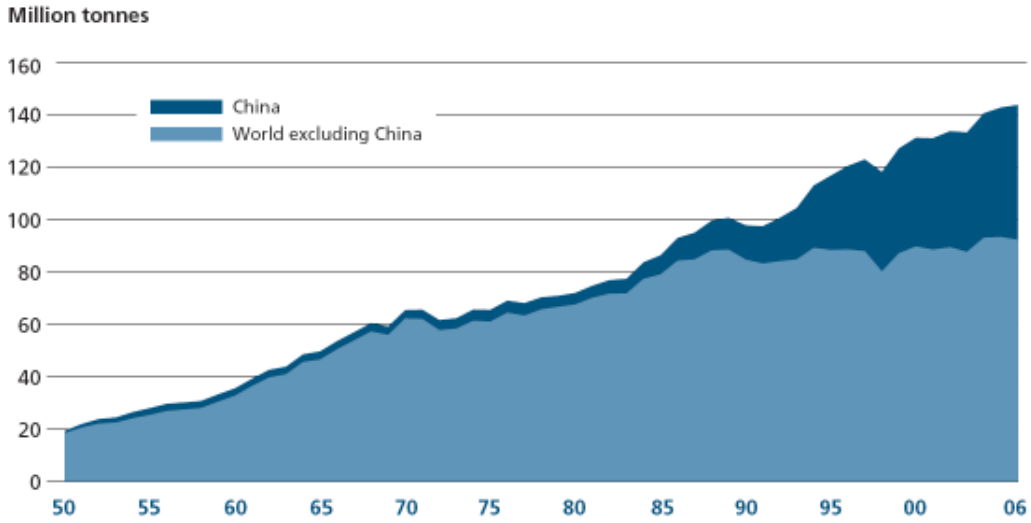


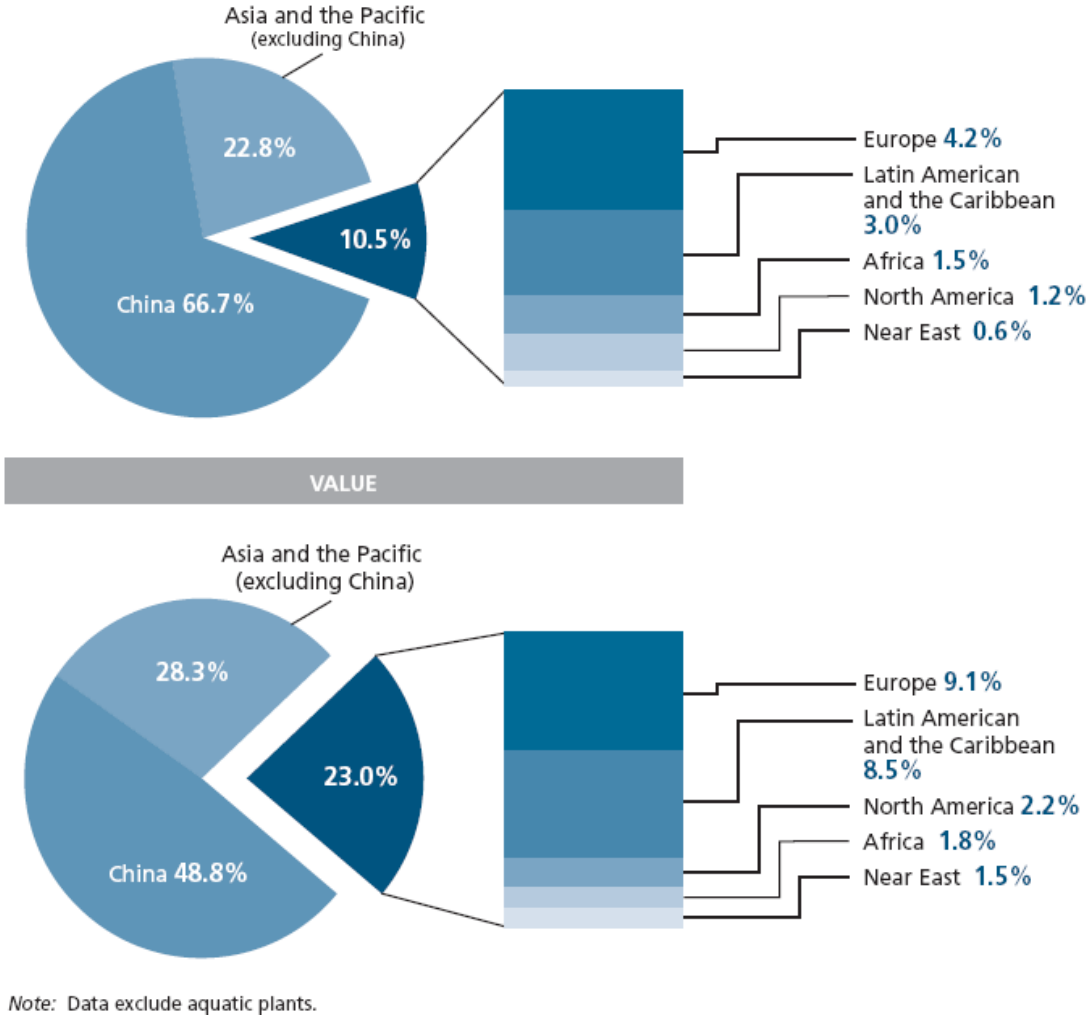
Figure 1. World fish production until 2006. Fish captures plus aquaculture production. SOFIA 2008.

Since more than 4000 years aquaculture has been used as a food source, although it's been the world's fastest growing food production system in the last three decades with a global production of fish, mollusks and crustaceans increased from 3.9% of total fishing production by weight in 1970 to 36% in 2006 (SOFIA 2008). Moreover, aquaculture is making an increasing contribution to fisheries production because of a backdrop of declining wild stocks with *per capita* supply from aquaculture increasing from 0.7 kg in 1970 to 7.8 kg in 2006 (an average annual growth rate of 7%). Globally, aquaculture account for 47% of the world's fish food supply in 2006. In China, 90% of fish food production comes from aquaculture, which indicates that aquaculture production in the rest of the world accounts for 24% of food fish supply (SOFIA 2008).

World aquaculture has grown dramatically in the last 50 years. From a production of less than 1 million tons in the early 1950s, production in 2006 was reported to have risen to

51.7 million tons, with a value of US $78.8 billion. This means that aquaculture continues to grow more rapidly than other animal food-producing sectors. While capture fisheries production stopped growing in around mid-1980, the aquaculture sector has maintained an average annual growth rate of 8.7% worldwide (excluding China, 6.5%) since 1970. Annual growth rates in world aquaculture production between 2004 and 2006 were 6.1% in volume terms and 11% in value terms.

Of the world total, China is reported to produce 67% of the total quantity and 49% of the total value of aquaculture production. An analysis of production by region for the period 1970–2006 (Figure 2) shows that growth has not been uniform. The Latin America and the Caribbean region shows the highest average annual growth (22%), followed by the Near East region (20%) and the Africa region (12.7%). China's aquaculture production increased at an average annual rate of 11.2 percent in the same period. However, recently, China's growth rate has declined to 5.8% from 17.3% in the 1980s and 14.3% in the 1990s. Similarly, production growth in Europe and North America has slowed substantially to about 1% per year since 2000. In France and Japan, countries that used to lead aquaculture development, production has fallen in the last decade. It is apparent that, while aquaculture output will continue to grow, the rate of increase may be moderate in the near future (SOFIA, 2008).



Note: Data exclude aquatic plants.

Figure 2. Aquaculture production by region. SOFIA 2008.

Most aquaculture production of fish, crustaceans and mollusks continues to come from inland waters (61% by quantity and 53% by value). An allocation of aquaculture production by aquatic environments shows that the freshwater environment contributes 58% by quantity and 48% by value. Aquaculture in the marine environment contributes 34% of production and 36% of total value (SOFIA, 2008).

This rapid increase in production was principally accomplished, during the centuries, thanks to mastery of reproduction of marine fish, the development of research in formulated diets and technological rearing innovations that permitted to establish aquaculture facilities both on land as well as in the sea.

According to FAO previsions, aquaculture seems to be a feasible complement to fisheries in order to satisfy the high demand of marine products. In 2004 worldwide fish consumption rate was 16.6 kg/person/year, Spain being one of the most important fish consumer's with 36.6 kg/person/year (Figure 3). Nevertheless, Spanish fish consumption depends on a fishing fleet that for many years has not been able to obtain the necessary captures to supply the national market (Roo, 2009).

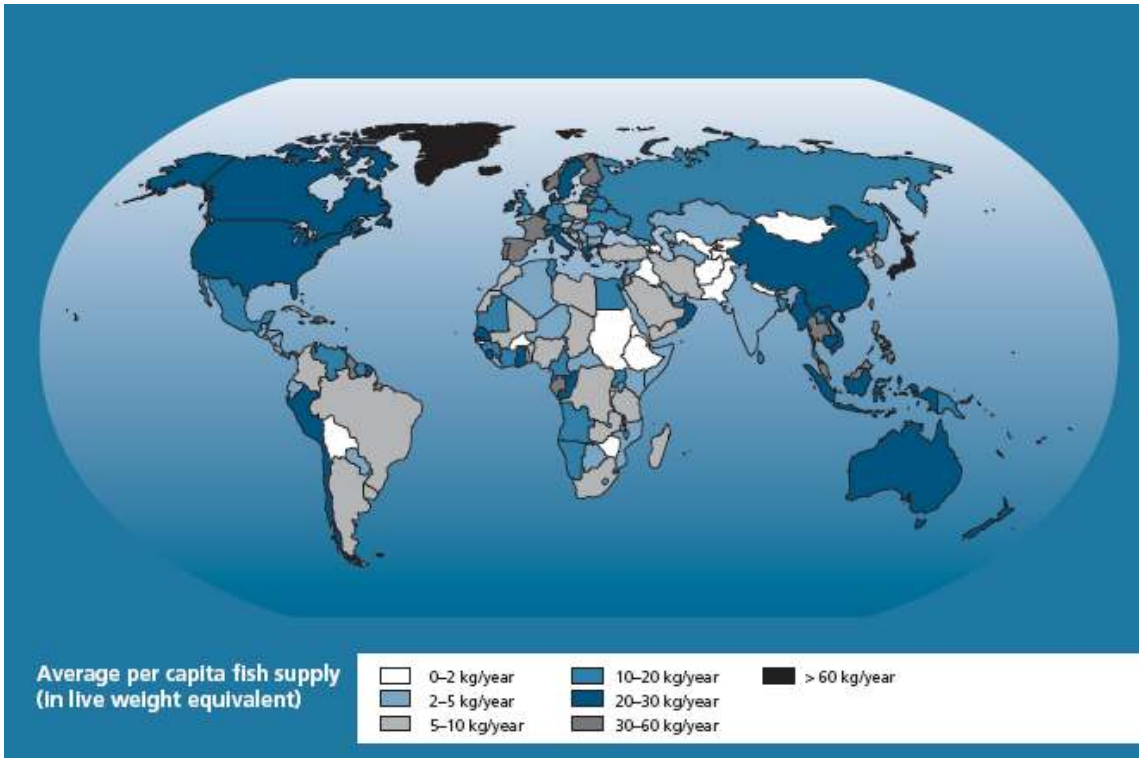


Figure 3. SOFIA, 2008 statistic world's fish supply.

Anyway, increased production requires increasing fingerlings supplies. Only for a limited group of species the production relies on hatchery outputs while for some other species, fingerlings supply rely exclusively on wild captures.

Another factor to be considered in the mass production of marine fishes is linked to the small size of the larvae and the need for small particle sizes which poses specific problems on the feed technology (Planas and Cunha, 1999). The production of marine larvae strongly depends on the reproduction of a simplified trophic chain, based on the use of microalgae, rotifers and Artemia. This situation implies the need to maintain parallel

facilities to fish larval tanks to produce live prey for larval feeding, increasing enormously the cost of fish fry (Roo, 2009).

Despite the recent progress in the production of inert diets for fish larvae, feeding of most species of interest for aquaculture still relies on live feeds during the early life stages. Independently of their nutritional value, live feeds are easily detected and captured, due to their swimming movements in the water column, and highly digestible, given their lower nutrient concentration with water content ≥80% (Conceiçao and Yúfera 2009).

The most commonly used live feeds in aquaculture are rotifers (*Brachionus sp*.) and brine shrimp (*Artemia sp*.), due to the existence of standardized cost-effective protocols for their mass production. However, both rotifers and Artemia have nutritional deficiencies for marine species, particularly in essential n-3 highly unsaturated fatty acids (HUFA, i.e., docosahexaenoic acid and eicosapentaenoic acid).

Several species of microalgae are also used in larviculture. These are used as feed for other live feeds, but mostly in the "green water" technique[1] in fish larval rearing, with putative beneficial effects on feeding behavior, digestive function, nutritional value, microflora balance and a general effect on water quality.

Nonetheless, technical difficulties in mass-producing these organisms are still a constraint to their routinely use. Improvements in inert microdiets will likely lead to a progressive substitution of live feeds. However, complete substitution is probably years away for most species, at least for the first days of feeding (Conceiçao and Yúfera 2009).

[1] The Green water method includes the generation of an endogenous trophic chain based in phytoplankton (*Nannochloropsis sp.*) and rotifers (*Brachionus sp.*) which are allowed to grow in filtered and sterilized seawater. Water is fertilized with nutrients and inoculated with phytoplankton and rotifers which developed until they reach a 500,000 phytoplankton cells.ml-1 and >2 rot.ml-1. The water is not renewed until day 4 to 8 when larvae are introduced. When the food chain is correctly developed this last approximately 25 days before *Artemia* and weaning diets are necessary.

1.1 Microalgae Production

Microalgae constitute the first link in the oceanic food chain, due to their ability to synthesize organic molecules using solar energy. In aquaculture, microalgae are produced as a direct food source for various filter-feeding larval stages such as bivalve mollusks (clams, oysters and scallops), some marine gastropods (abalone) and in the early stages of penaeid shrimps (Yúfera & Lubian, 1990). They are also used as an indirect larval food, because are used in the production of zooplankton (i.e., rotifers and Artemia), which in turn are the live preys for the carnivorous larvae of most of the marine fishes and shrimp species actually farmed. Moreover, also the intensive production of bivalves has, so far, relied on the production of microalgae, which comprises, on average, 30% of the operating costs in a bivalve hatchery.

In the "green water" fish rearing technique, algae are used directly into the larval tanks. This technique is nowadays a normal procedure in marine larviculture, given that it has been widely reported to improve fish larval growth survival and food ingestion. The observed larval quality enhancement, when using microalgae, has been showed in various studies, which demonstrated that microalgae seemed to provide nutrients directly to the larvae (Mofatt, 1981), contribute to the preservation of live prey nutritional quality (Makridis & Olsen 1999), promote changes in the visual contrast of the medium and to play an important role in the microflora diversification of both the tank and the larval gut (Skjermo & Vadstein, 1999).

More recently, Rocha, *et al*., 2008 showed that fish larvae feeding ability is also influenced by the presence of microalgae in the tank. However, this effect is not the same among species and has been shown to be more pronounced in gilthead seabream (*S. aurata*) than in Senegalese sole (*S. senegalensis*) larvae (Rocha, *et al.,* 2008).

Microalgae species were investigated in the present study based on their biological characteristics and performance under laboratory conditions, as well as on their nutritional and energetic properties. Among the most important selection criteria for microalgae, the following can be highlighted:

1. Cell size appropriate to the demands of the consumer organisms.
2. Adequate nutritional value.
3. High digestibility.
4. Easy culture at high densities.
5. Short life cycle, reproducible in captivity.
6. Tolerance to environmental variations.

Using these selection criteria, 16 genera of microalgae are nowadays produced. Still, some species dominate and it is possible to relate such species to their corresponding utilization. Class *Baccilariophyceae* (Diatoms) are usually given to bivalve molluscs and crustacean larvae as they are rich in silicates, which constitute their cell walls (frustules) and are necessary for bivalves and crustaceans for the formation of their rigid structures. Classes *Prasinophyceae* (e.g., *Tetraselmis suecica*, *Tetraselmis chuii*) and *Chrophyceae* (e.g., *Dunaliella tertioleta*, *Chrorella minutissima*) are ideal food for crustacean larvae, when complemented by *Baccilariophyceae* for silicate supply (Conceiçao and Yúfera 2009).

The growth of any axenic[2] culture of microalgae is characterized by five phases described in figure 4 (Lavens & Sorgeloos, 1996).

1. Lag or induction phase. This phase is characterized by a little increase in cell density. Cultures inoculated with exponentially growing algae have a short lag phase, which can seriously reduce the time required for up scaling. The lag in

[2] Not contaminated by or associated with any other living organisms. Usually used in reference to pure cultures of microorganisms that are completely free of the presence of other organisms.

growth is attributed to the physiological adaptation of cell metabolism to growth, such as the increase of the levels of enzymes and metabolites involved in cell division and carbon fixation.

2. Exponential phase. During this second phase, the cell density increases as a function of time according to a logarithmic function:

$$Ct = C0.e^{mt}$$

With Ct and C0 being the cell concentrations at time t and 0, respectively, and m=specific growth rate. The specific growth rate is mainly dependent on algal species, light intensity and temperature.

3. Phase of declining growth rate. Cell division slows down when nutrients, light, pH, carbon dioxide or other physical and chemical factors begin to limit growth.

4. Stationary phase. In the fourth stage growth limiting factors and the growth rate are balanced, which results in a relatively constant cell density.

5. Death or "crash" phase. During the final stage, water quality deteriorates and nutrients are depleted to a level incapable of sustaining growth. Cell density decreases rapidly and the culture eventually collapses. In practice, culture crash can be caused by a variety of reasons, including the depletion of a nutrient, oxygen deficiency, overheating, pH disturbance, or contamination. The key to the success of algal production is maintaining all cultures in the exponential phase of growth. Moreover, the nutritional value of the produced algae is inferior once the culture is beyond phase 3 due to reduced digestibility, deficient composition, and possible production of toxic metabolites.

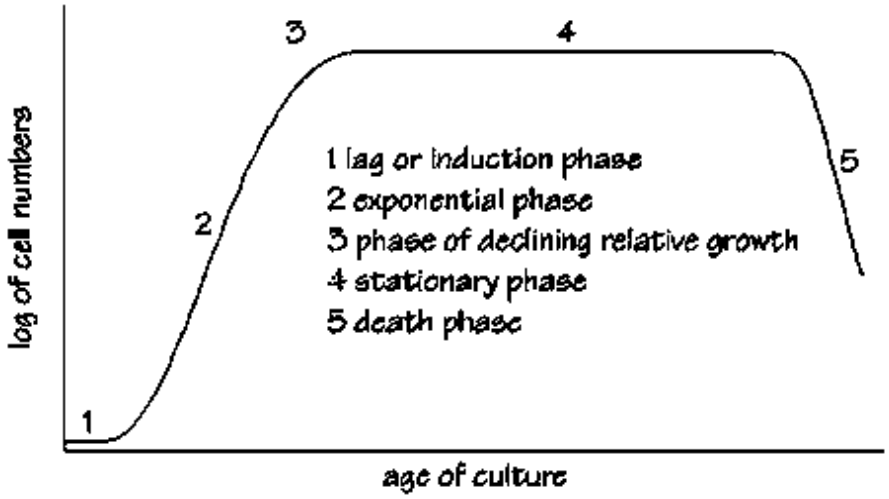


Figure 4. Culture phases of microalgae. Taken from Lavens & Sorgeloos, 1996.

According to the ICCM`s marine fish hatchery, the microalgae used for live prey feed and "pseudo green water"[3] system is *Nannochloropsis gaditana* (Lubian, 1982).

1.1.2 *Nannochloropsis gaditana*

Nannochloropsis gaditana (Table I) is a well appreciated alga in aquaculture due to its nutritional value and its ability to produce valuable chemical compounds, such as pigments (zeaxanthin, astaxanthin.) and polyunsaturated fatty acids (PUFAs). Anyway, the commercial exploitation of microalgae generally needs high cell densities but the low growth rate and the small size of *N. gaditana* cells are the principal bottleneck for the high scale production of this specie (Rocha, 2003).

Table I. Classification of *N. gaditana* (www.algaebase.org)

Taxonomic classification.	
Empire	*Eukaryota*
Kingdom	*Chromista*
Subkingdom	*Chromobiota*
Infrakingdom	*Heterokonta*
Phylum	*Heterokontophyta*
Class	*Eustigmatophyceae*
Order	*Eustigmatales*
Family	*Monodopsidaceae*
Genus	*Nannochloropsis*
Specie	*Nannochloropsis gaditana*

[3] This technique is based on an exogenous food chain, that contains phytoplankton and rotifers, produced in parallel facilities and that are daily introduced according to the larval demand

Size and morphology of microalgae are susceptible to variation due to culture conditions among their growth cycle. Indeed, when *N. gaditana* algae culture is in the exponential phase, the cells are ellipsoidal shaped 3.5-4µm x 2.5-3µm in size. In this circumstances *N. gaditana* can be distinguished from the other *Nannochloropsis* algae (Lubiàn, 1982). The algal cells are immovable, without flagella, with a pale color parietal simple chromophore which occupies a large part of the cell, they present a highly basophilic cytoplasm with lipid accumulation; The cell wall is a unique smooth piece as shown on figure 5.

The reproduction is exclusive by binary cell fission.

The chloroplast which occupies most of the cell contains a lamellae series formed by three parallel thylakoids each and a lack of envelope lamellae. The wall cell is covered by four membranes, two of which are external membranes and seems to correspond to the endoplasmic reticulum (Lubian, 1982).

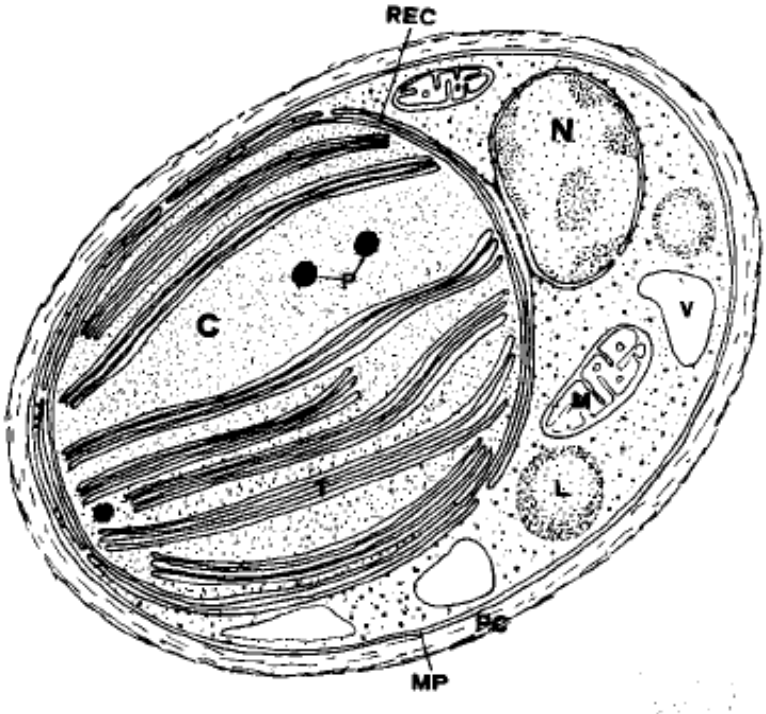


Figure 5. Principal characteristics of a *N. gaditana* cell structures. C.Chloroplast. MP. Plasmatic membrane. N. Nucleous. M. Mitochondria. REC. Endoplasmic reticulum. T. Thylakoids. PC. Cellular wall. V.Vacuoles. L. Lipids. P. Plastidial globules.

Microalgae can be cultivated in many different ways, depending on the purpose of the culture. The main microalgae culture systems may be described by these criteria:

- Indoors/Outdoors. Indoor cultures allow having control over environmental parameters such as illumination, temperature, pH, nutrient level, predator pollution and other competing algae. Outside cultures are very difficult to maintain pollution free for extended periods and most of the environment parameters can't be controlled.
- Open/Closed. Regardless of whether they are indoors or outdoors, open systems are more susceptible of pollution than closed containers.
- Axenic/xenic. Axenic systems are strictly closed due to avoid any kind of pollution through air filtration and sterilization of culture water, nutrients, containers and every tool used in the system plus a pure inoculation algae strain.
- Depending on the productivity of the system the microalgae culture can be distinguished in three groups, Batch culture, Semi-continuous and continuous culture.

1.1.3 Traditional culture

The batch culture is the most regular used at the Instituto Canario de Ciencias Marinas (ICCM) fish hatchery, the batch culture consists of a single inoculation of cells into a plastic bag with enriched water followed by a growing period of several days without manipulating the culture and finally harvesting when the algal population reaches its maximum/near maximum density. To scale up the culture and obtain a bigger volume of culture, algae are transferred to larger volumes prior to reach the stationary phase and the larger culture volumes are then brought to a maximum density and then completely harvested. Batch culture systems are widely applied because of their simplicity and flexibility.

Although often considered as the most reliable method, batch culture is not necessarily the most efficient. Batch cultures are harvested just prior to the initiation of the stationary phase and must thus always be maintained for a substantial period of time past the maximum specific growth rate. Equally, the quality of the harvested cells may be less predictable than that in continuous systems and for example vary with the timing of the harvest (time of the day, exact growth phase). Another disadvantage is the need to prevent contamination during the initial inoculation and early grow period. Because the density of the desired phytoplankton is low and the concentration of nutrients is high, any contaminant with a faster growth rate is capable of outgrowing the culture. Batch cultures also require a lot of labor to harvest, clean, sterilize, refill, and inoculate the containers (Lavens & Sorgeloos, 1996).

The semi-continuous method prolongs the use of culture through the partial periodic harvesting followed immediately by refilling up the original volume with nutrients plus water to achieve the original enrichment level. The culture is grown up again, and partially harvested and so on. Semi-continuous cultures may be indoors or outdoors, but usually their duration is unpredictable due to competitors, predators and/or contaminants and metabolites, that may make the culture unsuitable for further use. Since the culture is not harvested completely, the semi-continuous culture yields more algae production than the batch culture system.

In the continuous culture method, a supply of enriched seawater is continuously pumped into a growth container and the excess culture is simultaneously harvested. This system permits the maintenance of cultures very close to the maximum growth rate. Two categories of continuous cultures can be distinguished:

- Turbidostat culture, in which the algal concentration is kept at a preset level by diluting the culture with fresh medium through an automatic system.

- Chemostat culture, in which a flow of fresh medium is introduced into the culture at a steady, predetermined rate.

Open ponds can be categorized into natural waters (lakes, lagoons, ponds) and artificial ponds or containers (Figure 6). The most commonly used systems include shallow big ponds, tanks, circular ponds and raceway ponds. One of the major advantages of open ponds is that they are easier to construct and operate than most closed systems. However, major limitations in open ponds include poor light utilization by the cells, evaporative losses, diffusion of CO_2 to the atmosphere, and requirement of large areas of land. Furthermore, contamination by predators and other fast growing heterotrophs have restricted the commercial production of algae in open culture systems to only those organisms that can grow under extreme conditions. Moreover, due to inefficient stirring mechanisms in open cultivation systems, their mass transfer rates are very poor resulting to low biomass productivity (Ugwu, 2008).

As mentioned, closed systems offer major opportunities to avoid culture pollution and improve cell density; therefore closed photo-bioreactors provide a suitable option for microalgae culture taking advantage of small areas for high density algae cultures.

Figure 6. Commercial microalgae culture ponds. Kona, Hawaii.**h**ttp://www.algae4oil.com/_borders/clip_image001_000.jpg

1.1.4 Photo-bioreactors (Pbr)

A photo-bioreactor is a system, which provides an artificial environment to grow phototrophic cell cultures. Many factors influence the design of a successful photo-bioreactor such as light, carbon dioxide and nutrients. Light is often considered to be one of the most important factors (Richmond, 2004).

Closed photo-bioreactors have attracted much interest because they allow a better control of the cultivation conditions than open systems. With closed photo-bioreactors, higher biomass productivities may be obtained and contamination can be easily prevented (Xu, *et al.,* 2009).

The flat panel photo-bioreactors arise when one considers the laminar morphology of plant leaves, they are well evolved solar collectors, and thus flat panel type geometrics seem to have been modeled the same way with a high surface/volume ratio (Figure 7). Usually, the panels are illuminated mainly on one side by direct sunlight and have the added advantage that they can be positioned vertically or inclined at an optimum angle facing the sun (Xu, *et al.,* 2009).

These kinds of photo-bioreactors have been widely accepted for cultivation of photosynthetic microorganisms due to their big illumination surface area. Ramos de Ortega and Roux in 1986 developed an outdoor flat panel reactor by using thick transparent PVC materials; forming flat-plate photo-bioreactors made of transparent materials for maximum utilization of solar light energy. Accumulation of dissolved oxygen concentrations in flat-plate photo-bioreactors is relatively low compared to horizontal tubular photo-bioreactors (Ugwu, 2008).

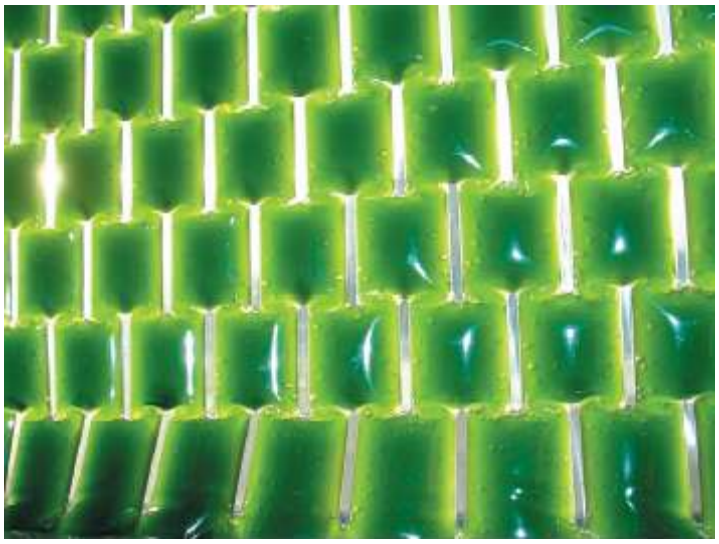


Figure 7. Flat-panel plastic photo-bioreactor. Geometric design for best solar collection. http://www.proviron.com

Various designs of photo-bioreactors have been tasted for algae mass cultivation one of which was vertical column (Figure 8). They have been considered due to their compact design, low cost production and easy operation, and also for very promising perspectives for large-scale production. Miron, *et al.,* reported in 2002 that bubble-column and airlift photo-bioreactors (up to 0.19 m in diameter) can attain final biomass concentration comparable to values reported for narrow tubular photo-bioreactors. Pneumatically agitated bubble columns and airlift devices attain the requisite mass transfer coefficient of 0.006 s^{-1} and liquid circulation velocity at a relatively low power input for practicable culture of microalgae (Miron, 2000).

Aeration rate is restricted by considerations of shear sensitivity. There is an upper limit on the acceptable level of turbulence, because algal species are affected differently by the hydrodynamic stress (Miron, *et al.,* 2000). Generally, increasing aeration rate increases mixing, liquid circulation, and gas-liquid mass transfer in bubble column and airlift bioreactors, and it also provides good gas-liquid mass transfer for efficient use of CO_2 (Xu, *et al.,* 2009). Not surprisingly some microalgal species also suffer negative effects from an increase in superficial gas velocity due to the high shear stress caused by high aeration rate.

Figure 8. Vertical column photo-bioreactors. Left. http://www.bioenergy-noe.com Right. ICCM`s fish hatchery vertical photo-bioreactors.

These vertical column photo-bioreactors possess some advantages for microalgae cultivation: fixed parts, low power consumption, high mass transfer rate, good solids suspension, homogeneous shear, rapid mixing, and due to the vertical orientation, less space is required for high density culture. Indeed, scale-up is quite sensitive to the penetration of light into the bioreactor which decreases exponentially with distance from the light source.

Among the proposed photo-bioreactors, tubular photo-bioreactor (Figure 9) is one of the most suitable types for outdoor mass cultures. Most outdoor tubular photo-bioreactors are usually build up with either glass or plastic tube and their cultures are re-circulated either with pump or preferably with airlift system. They can be in form of horizontal serpentine, vertical, near horizontal, conical or inclined photo-bioreactor (Ugwu, 2008).

Aeration and mixing of the cultures in tubular photo-bioreactors are usually done by air-pump or airlift systems. Tubular photo-bioreactors are very suitable for outdoor mass cultures of algae since they have large illumination surface area. On the other hand, one of the major limitations of tubular photo-bioreactor is poor mass transfer. It should be noted

that mass transfer (oxygen build-up) becomes a problem when tubular photo-bioreactors are scaled up. For instance, some studies have shown that very high dissolved oxygen (DO) levels are easily reached in tubular photo-bioreactors (Molina and Fernández, 2001).

Figure 9. Left. Horizontal tubular photo-bioreactor in ICCM fish hatchery. Right. Industrial tubular photo-bioreactor for biofuel production. http://brae.calpoly.edu/CEAE/images/biofuels3.gif

Tube diameter is limited (generally 0.1 m). Increasing tube diameter results in a decrease in the surface/volume ratio, and this factor has a strong impact on the culture. As the algae grow and increase in density, they begin to shade one another and this translates to a volumetric reduction in biomass per unit of incident light (Xu, *et al.,* 2009).

The tubes length is directly involved in the circulation and permanence time of the culture medium inside the bioreactor, that's why scaling up a tubular photo-bioreactor theoretically, can be made increasing the length or diameter of the tube. On the other side, increasing tube diameter is not desirable in order to avoid the increase of dark zones inside the tubes. Moreover, increasing the length of tubes has the disadvantage to allow the O_2 produced by photosynthesis to accumulate, probably causing cell photosynthesis inhibition. Indeed oxygen concentrations above 35 mg L^{-1} are toxic to most microalgae (Xu, *et al.,* 2009).

The increasing length of the tubes, can lead to the establishment of a decreasing CO_2 gradient along the tubes that may cause a no-homogeneous access to the carbon source by the algal cells. This gradient in carbon fixation can increase the pH in the culture up to 11.

On the other hand, inadequate fixation of CO_2 sprinkled into the culture medium may result in the acidification of the medium due to the formation of carbonic acid. A well functioning photo-bioreactor will have a well balanced pH; where the rising hydroxyl ion (HCO_3^-) produced from photosynthesis is counterbalanced by increased carbonic acid production from CO_2 sparging (Xu, *et al.,* 2009).

2. Objectives

2. Objectives

Improve the microalgae (*N. gaditana*) production inside the ICCM`s fish hatchery facilities by two different ways:

- Increase microalgae cell density in the traditional bag production systems inside the microalgae room injecting CO_2 gas and increase nutrient income.
- Increase microalgae cell density establishing two different kinds of outdoor photo-bioreactors in a reduced space.

3. Materials and methods

3. Materials and methods

These experiments were performed at the fish hatchery mesocosmo facilities at the ICCM located in Taliarte`s port in Gran Canaria, Spain. The microalgae used was *Nannochloropsis gaditana* (Lubián, 1982). The materials used where bought by a local hardware store or were part of the mesocosmo equipment.

3.1 Microalgae culture

The microalgae culture were grown at different scale volumes:

1. Bags of 50 and 230 L.
2. 50L Vertical Photo-bioreactors.
3. 30L. Horizontal Photo-bioreactor.

N. gaditana culture was made using filtered and sterilized (UV) sea water to get to a final salinity of 25 ‰. Reducing water salinity, avoided the presence of algae competitors such as ciliated organisms, bacteria or other algae species. Originally, marine water salinity was of 37 ‰. The proper quantity of fresh and marine water used (Table II) was calculated through the formula:

$$C_1 V_1 = C_2 V_2$$

Table II. Fresh and sea water volumes used to get to final culture salinity to 25 ‰

Final Water Volume (L)	Fresh Water (L)	Sea Water (L)
30	10	20
50	16	34
230	75	155

The culture medium used to enrich water was the Guillard and Ryther (1962) f/2 medium. Specifically a commercial product was used (Nutri-phyt, Easy algae – Spain)

made of two different solutions: The first one contained nitrates, phosphates, silicates and minerals; the second one is composed of vitamins. The first solution had to be kept in darkness (due to the presence of photo-oxidizing components) at room temperature; while vitamins solution was kept at + 4°C (to avoid component`s heat denaturalization). Regular nutrient dose is reported in table III.

Table III. Regular nutrient doses for microalgae cultures

Final Water Volume(L)	*Nutrients + minerals (0.2 ml(L)$^{-1}$)*	*Vitamins (0.05 ml (L)$^{-1}$)*
30	6	1.5
50	11	2.5
230	46	11.5

Before algae inoculation, the "mother bag"[4] was treated adding 0.05 ml (L) $^{-1}$ of formaldehyde 35-40% stabilized with methanol (Panreac Chemistry, Spain) to reduce possible algae competitors such as ciliates. The inoculation volume depended on the initial concentration desired in the new bag. The initial concentration was calculated evaluating cell concentration in the "mother" bag (where inoculum was taken off) applying the volume-concentration equation mentioned before.

The algal volume to be inoculated was added through a plastic funnel directly into the bags or photo-bioreactors. Algae initial concentration was fixed as day "0" (inoculation day).

3.1.1 Traditional bag culture

Experiments with this system were conducted for 10 days during the exponential growth phase plus 5 more days during the stationary phase. Semi continuous culture[5] began with a daily harvest at the 10th day, (late exponential phase) before algal culture did not show any global increase in concentration due to a lack of nutrients and the

[4] Mother bag is intended as the culture from which the inoculum was taken to start a new bag. Generally the former was at the stationary phase (i.e. when the multiplication rate of algal cells is equal to the its death rate).

[5] Semi-continuous culture is intended when an aliquot of each culture is harvested and supplanted with the same volume of enriched water.

accumulation of secondary metabolites. The experiments were done by sixfold, this means that every bag was considered as a replica of each treatment.

Plastic bags were prepared using transparent polyethylene of 800 gauges (thickness) of 50 x 100 cm. for the 50 L bags and 50 x 500 cm. for the 430 L bags (Figure 10).

Figure 10. Polyethylene plastic bags.

Before adding any liquid, the plastic bags where closed at both ends attaching them with two string supports to a steel holding structure. Once secured with plastic clamps (Figure 11 A), two holes were made at the superior sides of the bags where water was added (Figure 11 B and C). Each bag was provided with two air diffusers, one at each side and one CO_2 diffuser when needed (Figure 12). They were previously disinfected with common bleach and rinsed abundantly with sea water to neutralize the bleach.

A B *C*

Figure 11. A; string supports on the steel holding structure and bags attached to them with plastic clamps. B, holes in the plastic bag side to introduce culture components. C, water addition through a plastic hose.

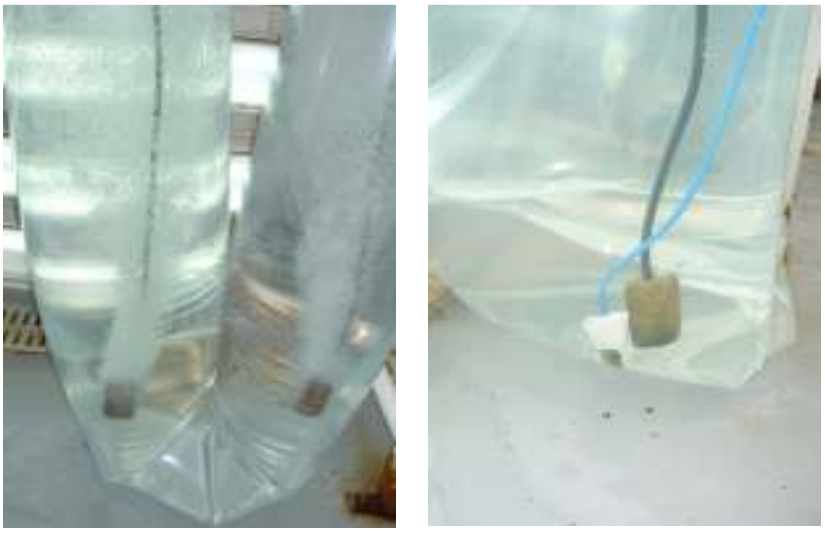


Figure 12. Left, one air diffuser on each side of the bag. Right, just one CO_2 diffuser (blue hose) per bag.

Algae inoculums and nutrients were added through the same holes in the bags (Figure 13 A and B).

Illumination was artificially provided, 24 hours a day, by 24 fluorescent light bulbs (Phillips TL-D 58W/54 - 765) twelve at each side of a set of six bags, this sets of light bulbs had a constant irradiance range between 8800 to 16400 lux. The quantity of light at the outward side of the bags was determined by weather conditions during day time (Figure13 C).

A B *C*

Figure 13. A. Addition of nutrients to each bag through the holes on the bag. B; Bag inoculation. C; Twelve fluorescent light bulbs on each side of the bag holding structure

3.1.2 Vertical Photo-bioreactors (VPbr)

Two vertical methacrylate tubes were set up as outdoor photo-bioreactors. Each tube was 180 cm tall with 20 cm. base diameter and 4mm thickness (Figure 14 A). The total volume of each cylinder was of 55 L. Each photo-bioreactor was covered with a PVC lid with a PVC elbow union installed at the top where water heater (Eheim 50 W., mod. Jager 3602, Germany), air and water hoses get into the tubes (Figure 14 B and C).

Culture medium and inoculums preparation were made as mentioned for traditional bag culture. The total algae volume was maintained at 50 L to prevent medium spilling due to air bubbling. The VPbrs where done with no replicates, they were established as single systems tested consequently one after the other. The VPbrs were equipped with a peristaltic pump (Iwaki, Japan) shown on figure 15 (A) for nutrient adding that was connected to a timer (Orbis, Spain) shown on figure 15 (B) that operated during daytime injecting enriched water from the 30 L water reservoir (Figure 15 C) to the VPbrs.

A B C

Figure 14. A; Vertical photo-bioreactor 55 L. methacrylate tubes. B. PVC lid with PVC elbow to introduce air and water hoses. C; 50 W. water heater.

A B C

Figure 15. A: Iwaki™peristaltic pump to add enriched water during sunlight hours. B; Orbis timer commanding the peristaltic pump to work during daylight hours. C; plastic container with enriched water.

3.1.2.1 System Operation

The outdoor vertical photo-bioreactors were exposed to natural sunlight. The air hose entered the VPbrs through the PVC elbow on the lid at the top and descends to the bottom of the tube where the air was released in big bubbles to maintain a turbulent flow inside the tubes. The water heater was directly set inside the tubes at 25º C.

As any microalgae culture, the system has to reach the late exponential phase, which means that on the very first ten days, the algae grew up to the exponential phase without manipulating the system. After reaching the late exponential phase at the 10th day, a 10%

of the VPbr culture was daily harvested (5 L) and the same aliquot of enriched water added.

The VPbr (Figure 16) functioned as follows:

1. The enriched water was pumped through the peristaltic pump (Iwaki electromagnetic metering peristaltic pump. Model EW-F31VC-20EPF5, Japan) connected to the timer (Orbis, Spain) set to operate during eight daylight hours with a rate of 10.42 ml $(min)^{-1}$.

2. The air hose supplied big bubble aeration to keep turbulent flow inside the tubes and prevent sedimentation or stuck algae at the tube walls. The water heater was settled in order to start working when the culture temperature descended beneath 25° C, (most of the times it just worked at night).

3. The algae were passively collected in a plastic container at the same rate that the enriched water entered the system.

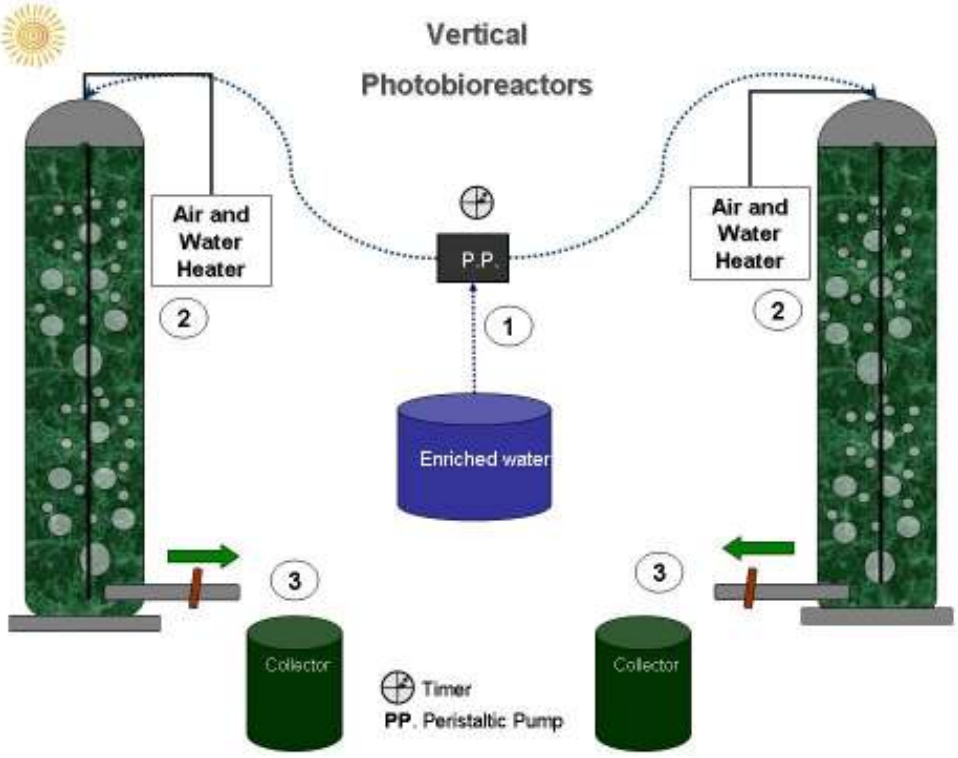


Figure 16. Vertical Photo-bioreactors system. 1) Enriched water pumped into the methacrylate tubes. 2) Air supply and water heater are installed inside each tube. 3) Plastic drums to collect the daily harvest production.

3.1.3 Horizontal Photo-bioreactor (HPbr)

This algae production system was made as a single system with no replicates in due of the material availability, this system consisted in two different parts; one located outside at the natural sunlight, and a second one inside the mesocosmo unit.

In the inside equipment the inoculum was made of a previous Pbr *N. gaditana* culture with a higher cell concentration; the culture medium was inoculated directly in the 50L black glass fiber tank (Figure 17 A). The water chiller (One hp. homemade chiller) was installed next to the fiber glass tank with a 5m plastic serpentine which goes inside the tank through the tap (Figure 17 B). The chiller was set on 14°C., to maintain the culture water close to 25°C., during the warmest hours of the day (Figure 17 C).

Also the water heater (Eheim 50 W., mod. Jager 3602, Germany) was installed inside the same glass fiber tank and set on 25°C., to maintain warm the water culture during the night (Figure 18 A).

A B C

Figure 17. A. Glass fiber black recipient (50L.) where the liquid-gas mass transfer takes place, also temperature and pH were controlled. B, automatic homemade water chiller next to the black glass fiber tank. C. plastic serpentine of 5 m. long, where cold water circulates from the chiller to the inside of the black tank for cooling water due to heat transfer.

The pH sensor was inside the glass fiber tank too connected to the pH controller (Milwaukee™ mod. SMS122, U.S.A.) on an electric panel board installed on the wall. This pH controller has an electro-valve included with the Milwaukee™ pH controller (Figure 18

B and C) situated on the CO_2 gas cylinder (200L) and a 2 mm. diameter plastic hose from the valve to the pipe just before the water pump (Figure 19 A and B).

A B C

Figure 18 A. Water heater (50 W.) settled up at 25 Celsius degrees inside the black glass fiber tank. B. Milwaukee pH controller and pH sensor, the sensor was located inside the black glass fiber tank. C. Milwaukee automatic electro-valve connected to the CO_2 cylinder on one side and to the pH controller on the other.

The peristaltic pump (Iwaki™ electromagnetic metering peristaltic pump. Model EW-F31VC-20EPF5, Japan) was located on the shelf above the fiber glass tank connected on one side to the plastic water reservoir container (Figure 19 C) and on the other side to the fiber glass tank with a none return valve.

A B C

Figure 19. A. CO_2 gas cylinder with the electro-valve and plastic hose on the gas outlet. B. Plastic hose from the CO_2 electro-valve connected to the water pipe after the black tank and before the water pump. C. Enriched water plastic container connected to the peristaltic pump which leads the water to the fiber glass tank.

The 20 methacrylate transparent tubes, 200cm long and 2 cm diameter were installed outside the mesocosmo facilities for sun irradiance absorption, this tubular structure was

assembled in a staged way with PVC elbow unions in a PVC support for the tubes. These tubes were connected with the water pump located indoors through the wall with a 20 mm diameter plastic hose (Figure 20 A, B and C).

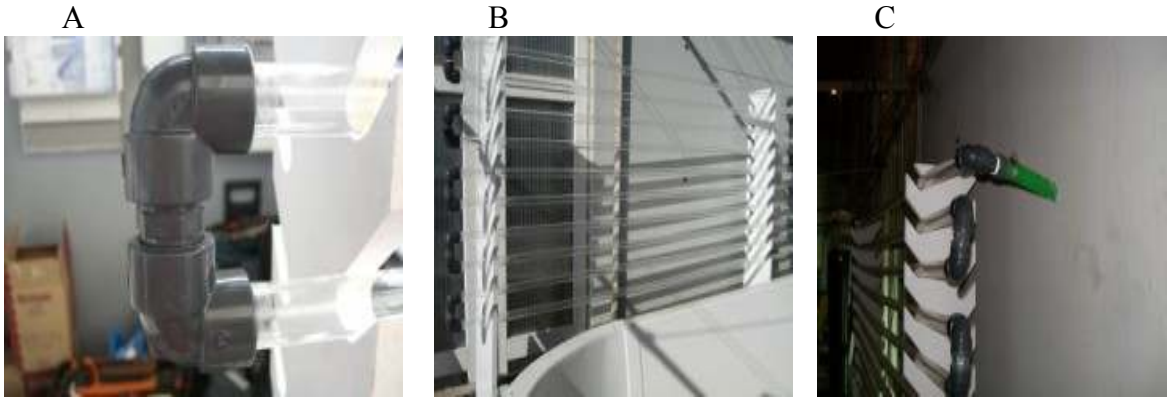


Figure 20. A. Elbow PVC unions for staging the methacrylate tubes. B. PVC supports for the 20 methacrylate tubes located outside the ICCM hatchery. C. Plastic hose connecting the outside tubes, with the inside water pump and equipment.

3.1.3.1 System Operation

As any microalgae culture, the system has to reach the late exponential phase, which means that on the very first ten days, the algae grow through the exponential phase without manipulating the system. The HPbr (Figure 21) works as follows:

1. The HPbr, system starts on the dark glass fiber tank where the microalgae flow from the bottom tap through a 20 mm diameter plastic hose connected to the water pump

2. The water pump sends the microalgae to the exterior traveling 40 lineal meters through the tubular structure composed of 20 methacrylate tubes (620 ml each) at 14.4 L $(min)^{-1}$ rate.

3. The microalgae culture return inside the dark tank where the liquid-gas mass interchange takes place.

4. The pH sensor inside the tank sends a signal to the pH controller when the pH values descend beneath 7.5 opening automatically the electro-valve located in the CO_2 cylinder allowing the gas flow through the pipe just before the pump send the microalgae

to the outside tubular panel where CO_2 is well used in the algae photosynthesis before it returns to the dark tank and degasification occurs. When the pH value return to 7.5 the electro-valve automatically close and stops the CO_2 gas flow.

5. Culture medium temperature is constantly controlled inside the dark tank with the water chiller and heater mentioned before.

After reaching the late exponential phase at the 10th culture day, 10% of the HPbr volume enriched water was daily added and harvested (3 L).

6. The enriched water was pumped through the peristaltic pump connected to the timer (Orbis, Spain) settled to operate just during eight daylight hours with a rate of 6.25 ml (min)$^{-1}$.

7. The algae were passively collected in the same rate that the enriched water was added to the system.

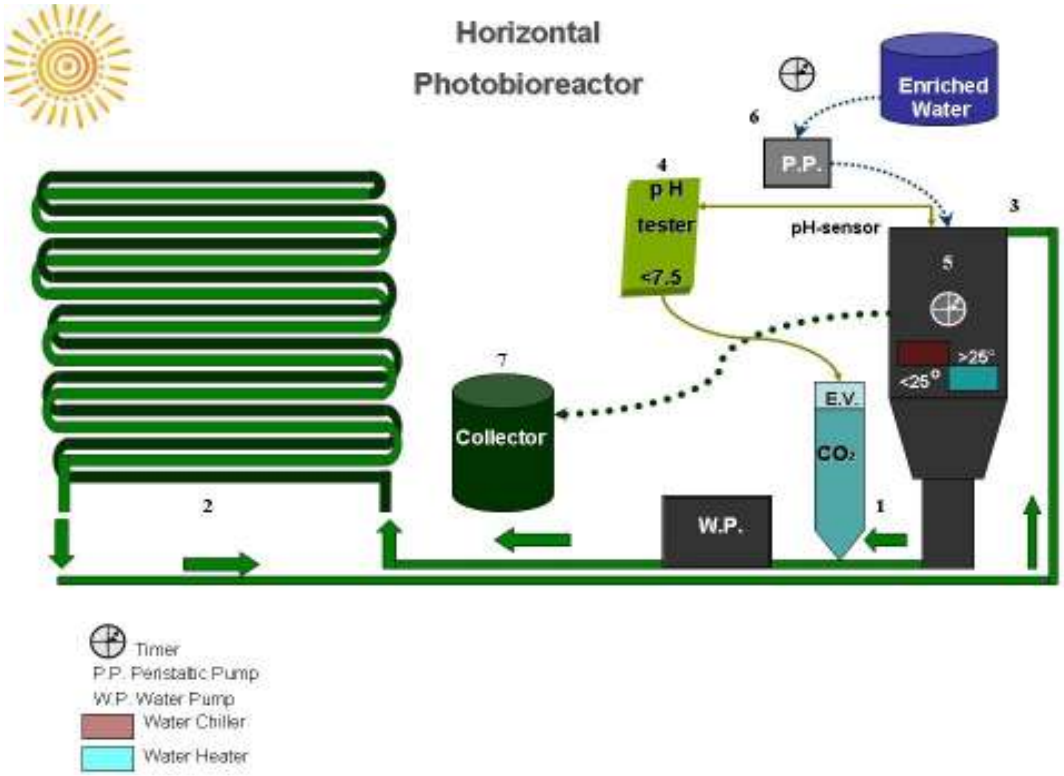


Figure 21. Components of the Horizontal Photo-bioreactor. Diagram shows the path that algae makes in the system circuit. The tubular panel is located outdoors and all the remaining system is located indoors.

3.2 Biological and chemical parameters monitoring

The microalgae cell concentration growth and chemical and physical parameters were daily evaluated. The microalgae samples were taken on the exponential or stationary growth phase depending on each experiment requirements.

The counting cell method was necessary for a correct growth evaluation of the culture development. Individual cell count was made in an improved Neubauer counting chamber under the optic microscope (Olympus CX21, Germany) using the 400X objective. The algae samples were taken from all the algae systems using a 1 ml pipette letting one drop into the counting chamber with a slide cover over it. When the cell concentration is higher enough to hinder the count, the sample was diluted 1:10 for a better appraisal of the cell density.

The counting chamber is made up of two different counting parts divided into different surfaces (squares) as shown on figure 22. Chamber depth is 0.1mm and each square has a 0.04 mm^2 surface area. So, the cells were counted in four 0.004 mm^3 (0.04 mm^2 x 0.1 mm) equals to a total volume of 0.004 µL. The mean of this four counted squares was obtained and divided between the square volume and multiplied by 1000 (conversion factor from µL to ml), the resulting value was expressed in million cells $(ml)^{-1}$ as shown in the next equation:

$$\left[\frac{Cells \times 10^6}{mL.}\right] = \frac{X(mean)}{0.004 \mu L.}(1000 \mu L.)$$

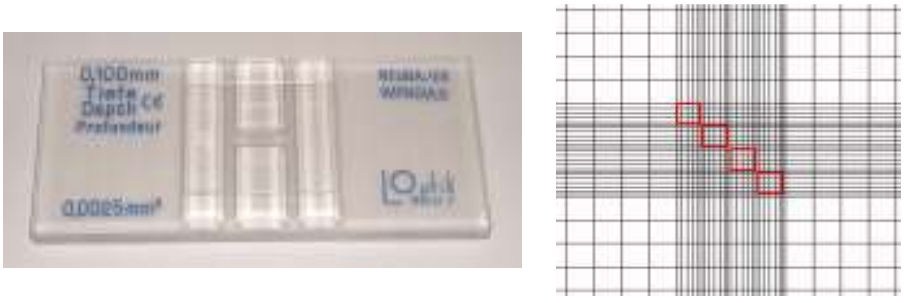


Figure 22. Left. Neubauer improved counting chamber. Right. 4X4 squares counting surface on each side of the chamber.

Another algae growth evaluation method was using the water turbidity. This is a fast and simple way to estimate the total biomass using a simple photometer. The measure unit is the FAU (Photo Absorbance Units) and it should be highly correlated with cell concentration values.

The photometer used (macherey-nagel pf-11, Germany) as show in figure 23 has a sensibility of 10 to 400 FAU, when this values are exceeded the sample has to be diluted until the FAU values fit the mentioned range and the resulting value should be recalculated multiplying by the dissolution factor.

Algae samples were collected in 10 ml crystal tubes, these tubes had to be very clean on their surface to avoid mistaken values due to elements that may affect the light reflection on the tube surface such as hand grease. The photometer has to be settled in "Nanocolor method" on the" turbidity" mode. A blank is made using clean seawater, after setting the blank each sample is read by the photometer using the filter number six as the photometer requires it for turbidity.

Figure 23. Macherey-Nagel compact photometer pf-11 used to calculate the water turbidity due to cell concentration.

Every day temperature and pH were directly measured; temperature was measured using a traditional mercury thermometer (Terimber 2284, Germany) and pH was measured using a pH-meter (Oakton Inc. Double junction pH Testr 3+, USA) directly into the algae sample.

Oxygen parameters were daily measured at 09:00 hrs with a commercial oxygen sensor (Oxyguard model Handy Polaris H01P, Denmark) shown on figure 24.

Figure 24. Oxyguard oxygen sensor.

Sunlight illumination (intensity) was daily measured at noon directly on the Pbr´s and bag surfaces with a digital light meter (HT170N,HT Instruments, Spain) shown on figure 25 The fluorescent irradiance in the bag cultures was constant on the side of the bags facing the fluorescent bulbs.

Figure 25. Digital light meter HT 170N used for Light irradiance measures.

To estimate the dissolved nitrate concentration in the microalgae medium, the cells had to be separated from the culture water, the sample of 130 ml was filtered through a 25 µm net and centrifuged for 5 minutes at 3000 RPM´s. The supernatant was removed and used for chemical analysis. To make the nitrate test, 5 ml of the supernatant were mixed with five

drops of part A plus one spoon of part B test reagents, after five minutes the sample was read in the compact photometer using the same supernatant as blank solution.

Macherey-Nagel Visocolor® nitrate test kit (Germany) (Figure 26) allows doing a photometric determination when nitrate is reduced to nitrite with an inorganic reducing agent. Nitrite is then diazotised with an aromatic amine and simultaneously coupled to form an azo dye which can be esteemed using the Macherey-Nagel compact photometer PF-11 settled on Nitrate mode with a detection range from 4 – 120 mg (L).

Figure 26. Macherey-nagel nitrate test kit.

3.3 Microalgae sampling and proximal analysis

Algae samples must be separated from water for biochemistry analysis. In order to get more than one gram of algae paste of each sample three litters of bag algae culture or two litters of Pbr algae culture were collected, filtered through a 25 μm net and centrifuged (Sigma 3K20, Germany) for 5 minutes at 3000 RPM (Figure 27). The supernatant was removed and the algae paste was washed twice with hydrochloric acid 0.1N (Panreac Chemistry, Spain) and twice rinsed with distilled water, the final paste was kept in plastic bags frozen (-80°C.) until the analysis was made.

Biomass production (g $(L)^{-1}$) was evaluated obtaining the weight of each cell (cw) after centrifuge a known volume of microalgae culture from each system. The biomass was obtained applying the next equation:

$$g(L)^{-1} = \text{cell density } (x10^{6} \text{ cells } ml^{-1}) * cw$$

Where cw = $1.03x10^{-8}$ for culture bags, $1.9\ x10^{-8}$ for VPbrs and $4.2\ x10^{-9}$ for HPbr.

Figure 27. A. Sigma centrifuge used to separate algae cells from water. B. Algae sample flasks before and after centrifugation. C. Algae pellet after removing supernatant

All samples were analyzed at the Aquaculture department laboratory in the IUSA (Instituto Universitario de Sanidad Animal) of the ULPGC (Universidad de Las Palmas de Gran Canaria, Spain).

The biochemistry analysis was made by triplicate (except when the algae sample was not enough, duplicate was done), this analysis included moisture, protein, lipid, carbohydrate and ash content, also its fatty acid composition was determined.

3.3.1 Ashes

The ash content was determined after incineration of a well-known amount of sample (Pm) in a Muffla oven, at 450ºC during 24 hours, remaining ashes amount was recorded (Pc) and weight until constant weight according to the AOAC (1995). Final ash content was obtained applying the following expression:

$$\%Ash = (100 \times Pm)/Pc$$

3.3.2 Moisture

Moisture content was determined by thermal drying to constant weight in an oven at 110 ºC, with a first 24 h drying period, followed by 1 h periods until weight was not reduced any further. Sample weight was recorded before drying and after each drying period, following the cooling in a desiccator to weight always at the same temperature. Moisture was expressed as a

percentage of the weight according to Official Methods of Analysis (A.O.A.C., 1995), using the following equation:

$$\text{Moisture content \%} = (100\ (B - A) - (C - A))/\ (B - A)$$

Where:

A= weight of empty flask

B= weight of wet sample + flask

C= weight of dry sample + flask

3.3.3 Proteins

Protein analysis was done according to Kjeldahl Method (A.O.A.C., 1995), which measures the total nitrogen content in the sample, and converting this figure to a total crude protein value by multiplying by the empirical factor 6.25.

3.3.4 Lipids

Lipids were extracted following the method of Folch *et al*., (1957). Weight samples of microalgae (0.1-0.2g) were homogenised in 10 ml chloroform: methanol (2:1 v:v) mixture. Lipid was then separated by centrifugation during 5 min (2000 rpm), the lower chloroform phase containing the lipid was carefully removed and evaporated to obtain lipid weight.

3.3.5 Fatty acid methyl esters analysis and quantification

Total lipids were dissolved in toluene and fatty acid methyl esters obtained by transmethylation with 1 % sulphuric acid in methanol (Christie, 1982). The reaction was conducted in dark conditions under nitrogen atmosphere for 16 h at 50º C. Afterwards, fatty acid methyl esters were extracted with hexane: diethyl ether (1:1 v/v) and purified by adsorption chromatography on NH_2 Sep-pack cartridges (Waters S.A., Massachussets, USA) as described by Christie (1982). Once separated the organic from the water phases fatty acid metil esters were diluted with HPLC hexane and quantified by gas (Termo Finnigan Fucus GC, Milan, Italy)

3.3.6 Microalgae bacteriology

Bacteriolgical analyses were made on *N. gaditana* cultures from the third microalgae improvement experiment. All bacteriology tests were done by triplicate at the Bacteriology laboratory at the ICCM, located in Taliarte`s port in Gran Canaria.

Samples of 100 ml were harvest directly from the different cultures (vertical and horizontal photo-bioreactors, bags) and kept in sterile flasks. The samples were immediately diluted (1:10) with sterilized distilled water ready to be inoculated in different specific mediums.

Before inoculation, important information was written on the bottom of the plates, close to the rim such as date of inoculation, dilution rate, temperature of incubation, duration of incubation and sample origin.

3.3.6.1 Enriched agar medium characteristics and preparation

Two different kinds of commercial enriched mediums were used to evaluate the bacteriological load and used depending on the performed tests (listed below).

TSA agar. Tryptic Soy Agar (Difco, Spain)

Medium formula per liter:

Tryptose (20g.)

Dextrose (1g.)

Disodium Phosphate (2g.)

Potassium Nitrate (1g.)

Agar (1g.)

Casein and soy peptones are a source of nutrients required for the replication of microorganisms. Sodium chloride maintains osmotic equilibrium. Lecithin and polysorbate 80, two commonly used neutralizers, are reported to inactivate residual disinfectants when the sample is being collected. Lecithin is incorporated to neutralize quaternary ammonium

compounds and polysorbate 80 is used to neutralize substituted phenolic disinfectants. Agar is the solidifying agent.

The agar preparation was made as follows:

An aliquot of 40 g of the powder was suspended in one litre of purified water and mixed thoroughly then heated with frequent agitation and boiled for one minute to completely dissolve the powder. The mix was autoclavated at 121°C for 15 minutes. Then the molted medium was poured into a sterile plastic Petri dish allowing the medium to solidify. The plates were stored and protected from light, in an inverted position (agar side up) at 2-8°C before use.

The inoculation procedure was made as follows:

The previously diluted sample was filtered through a 0.45μm pore size polycarbonate sterilized filter (Millipore Corp., U.S.A.). The filter was placed in TSA agar Petri plate and incubated 24 hours at 26°C for visible direct viable count (DVC) analysis under a stereomicroscope (Cambridge245 E, UK.).

TCBS Agar Thiosulfate Citrate Bile Salts Sucrose Agar (Difco Spain).

Medium formula per liter:

Yeast Extract (5g.)

Proteose Peptone No. 3 (10g.)

Sodium Citrate (10g.)

Sodium Thiosulfate (10g.)

Oxgall (8g.)

Saccharose (20g.)

Sodium Chloride (10g.)

Ferric Ammonium Citrate(1g.)

Bromthymol Blue (0.04g.)

Thymol Blue (0.04g.)

Agar (15g.)

TCBS is used for the selective isolation of cholera vibrios and *Vibrio parahaemolyticus* as well as other vibrios. Inhibition of gram-positive bacteria is achieved by the incorporation of oxgall, which is a naturally occurring substance containing a mixture of bile salts, and sodium cholate, a pure bile salt. Sodium thiosulfate serves as a sulfur source and, in combination with ferric citrate, detects hydrogen sulfide production. Saccharose (sucrose) is included as a fermentable carbohydrate for the metabolism of vibrios. The alkaline pH of the medium enhances the recovery of *Vibrio cholerae*. Thymol blue and bromthymol blue are included as indicators of pH changes.

The agar preparation was made as follows:

First, 89 g of the powder where suspended in one liter of purified water and mixed thoroughly, then heated with frequent agitation and boiled for one minute to completely dissolve the powder. Then cool to 45-50°C and use immediately. Did not autoclaveted. To prepare plated media, agar deeps with caps loosened where placed in a boiling water bath until the medium became liquefied, and then the molten medium was poured into a sterile Petri dish allowing the medium to solidify.

The plates where stored and protected from light, in an inverted position (agar side up) at 2-8°C until it was ready to use.

The most common method of inoculating a TCBS agar plate is streaking. With this method, a small amount of the sample is placed on the side of the agar plate (as a drop from the liquid sample).

A sterile loop (flamed until red hot, then cooled by touching the agar away from the inoculated sample) is then used to spread the bacteria out in one direction from the initial site

of inoculation. This is done by moving the loop from side to side, passing through the initial site (Figure 28).

The loop is then sterilized (by flaming) again and the first streaks are then spread out themselves. This is repeated 2-3 times, moving around the agar plate. The plates were incubated at 35 Celsius degrees for 24 hours.

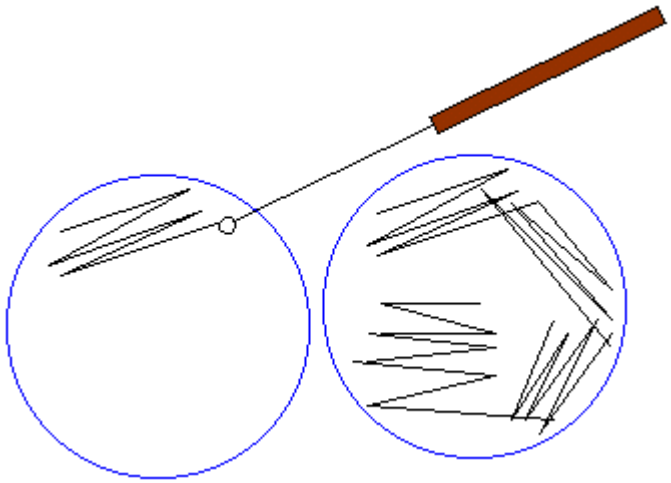


Figure 28. Streak plates inoculation. Picture: http://openwetware.org/wiki/Streak_plates

When the color of the TCBS plate changed from green to yellow or green blue due to the presence of possible *Vibrium* sp. colonies, the most abundant colonies (based on the widest surface coverage) were isolated from the plate and inoculated in another TSA plate to promote the isolated strain to grow and tried their identification as shown in figure 29.

Figure 29. Left.TCBS plate after incubation, some colonies appeared and turn yellow the medium. Right. The yellow colonies were isolated from the TCBS medium plate into another TSA medium plate.

3.3.6.2Visible Direct Count (VDC)

After 24 hour incubation, TSA agar Petri plates containing inoculated filter were opened and located under stereomicroscope for a visible direct colony count (Figure 30). Results were reported as colony former units per milliliter after the proper dilution calculation (CFU·ml.$^{-1}$).

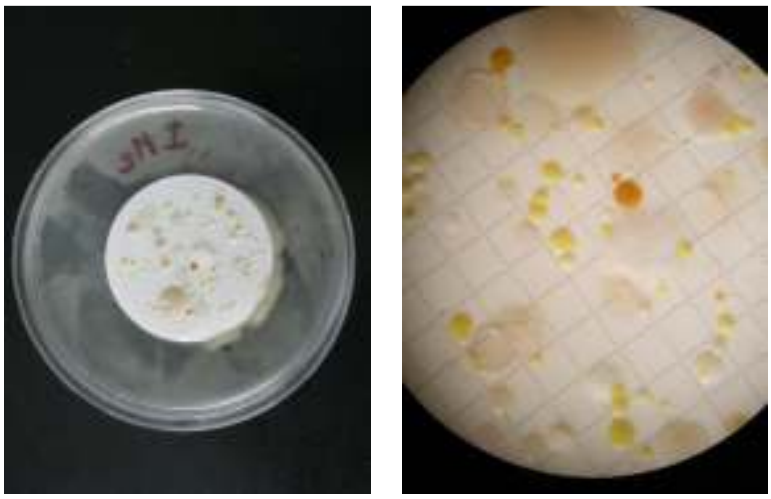


Figure 30. Left. Filter on Petri plate alter incubation. Right. Filter counting surface under the stereoscopicmicroscope.

3.3.6.3 Strain identification tests

In order to identify specific bacteriological strains that compose the microalgae culture, five different tests were done in the last microalgae experiment.

The oxidase test is used to determine if an organism possesses the cytochrome oxidase enzymes. The test was used to differentiate pseudomonades from related species. The cytochrome system is usually present in aerobic organisms which are able of utilizing oxygen as the final hydrogen receptor and it´s not present in faculty anaerobic bacteria. The end product of this metabolism is either water or hydrogen peroxide. The test reagent *N, N, N´, N´*-tetra methyl-*p*-phenylenediamide dihydrochloride (Sigma Chemicals Co.) acts as an artificial electron acceptor for the enzyme oxidase. The oxidase reagent forms the colored compound indophenol blue.

The cytochrome oxidase test was made by the impregnated strip method done as follows:

A fresh colony growth was scraped from the Petri plate with a disposable loop and rubbed into the filter paper (Remel Inc., U.S.A.) as shown on figure 31. The paper was examined for blue color after 10 seconds.

A B C

Figure 31. A. Scarping fresh colony from Petri plate. B. Oxidase strip test. C. Blue color after 10 seconds.

The catalase test is also one of the main three tests used to identify species of bacteria. The presence of catalase enzyme in the test isolated is detected using hydrogen peroxide. If the bacteria possess catalase (is catalase positive), when a small amount of bacterial isolate is added to hydrogen peroxide, bubbles of oxygen are observed.

The catalase test was done by placing a drop of hydrogen peroxide on a microscope slide. Using an applicator stick, a colony was scraped from the Petri plate and then smeared into the hydrogen peroxide drop.

If bubbles or froth forms, the organism was catalase-positive, if not, the organism is catalase-negative as shown on figure 32.

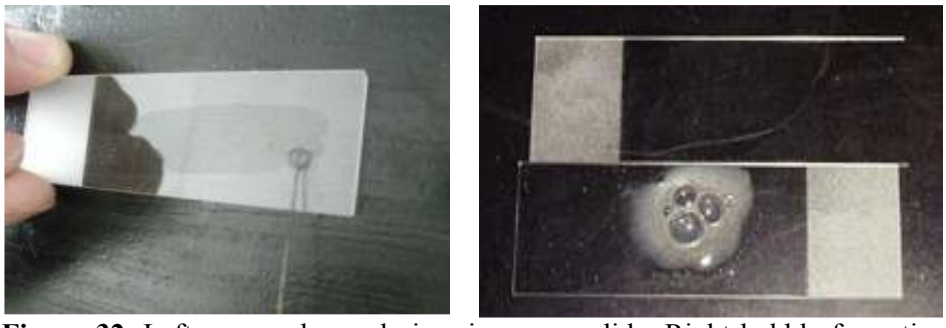


Figure 32. Left, smeared sample in microscope slide. Right bubble formation after hydrogen peroxide drop (catalase positive).

The motility test was used to determine if an organism is motile or non-motile. Motile organisms are generally bacilli (by means of flagellum) although a few motile cocci are known. This test was made by the hanging drop method which was made as follows:

A small drop of liquid bacterial culture was placed in the center of a coverslip with a drop of water in the coverslip corner, and then a slide with a central depression was placed inverted over the coverslip which got stuck to the slide and the bacteria culture suspended well with the water drop. The slide was turned arround and the coverslip sealed with paraffin. The slide was placed under the microscope (100X) and examined for motile organisms[6].

The Gram stain protocol involves the application of a series of dyes that differently stain bacteria in purple (Gram +) and pink (Gram -).

The different stain is due to the properties of the external bacterial cell wall. The purple color of Gram positive bacteria is due to the presence of peptidoglycans external to their cell membrane that retains the primary stain (crystal violet). On the contrary, Gram negative bacteria presented the peptidoglycan inside the cell membrane, therefore, they do not retain the primary dye but only the secondary one (safranine red) appearing pink. The staining procedure was done as follows:

First a bacterial smear was prepared. The microscope slide was then rapidly dried by heat on a Bunsen flame. Heat allowed the bacteria to stick on the slide fixing the sample to be stain. The slide was then flooded with Crystal Violet (the primary stain) for one minute and then rinsed in distilled water.

The slide was then flooded with iodine[7] for one minute and then rinsed with water.

Following, the slide was flooded for 10-15 seconds with a 1:1 acetone-ethanol mix[8]., and then rinsed in distilled water.

[6] Motile organisms appear to cross the entire visual camp, quickly from side to side.
[7] Iodine is a mordant that binds with Crystal violet and is then unable to exit the Gram (+) peptidoglycan cell wall.
[8] Ethanol is a destaining product that removes the stain from the Gram-negative cells.

At last the slide was flooded with Safrinin red (the secondary dye), and then rinsed with distilled water (Figure 33 A, B and C). Slides were viewed under an optic microscope (Olympus CX21) with immersion oil at a magnification of 100 X to examine the color of the bacteria as shown in figure 34.

A B C

Figure 33. A. Smear of the sample on a microscope slide. B. Addition of safrinin red. C. Wash procedure to eliminate the excess of dyes.

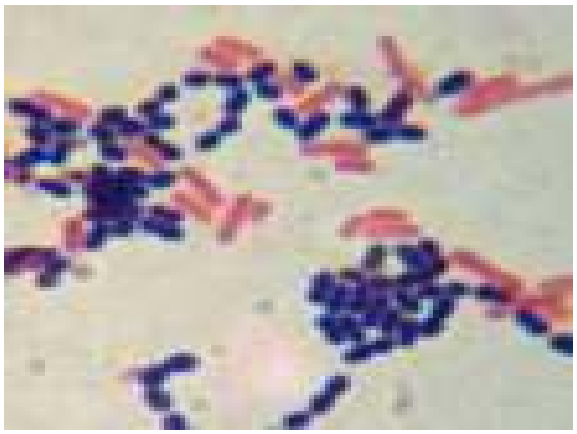


Figure 34. Staining of Gram + (purple) and Gram – bacteria (pink).

The API 20E™ (BioMerieux, France) identification test kit is a standardized identification system for *Enterobacteriaceae* and other non-fastidious Gram negative rods which uses 21 miniaturized biochemical tests and a database.

The API 20 E strip consists of 20 microtubes containing dehydrated substrates (Figure 35). These tests are inoculated with a bacterial suspension that reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents.

The reactions are read according to the Reading table and the identification is obtained by referring to the Analytical Profile Index (Included on API 20E test kit).

The microorganisms to be identified must first be isolated on a culture medium adapted to the culture of *Enterobacteriaceae* and/or non-fastidious Gram-negative rods, according to standard microbiological techniques.

Figure 35. API 20E strips, Analytical Profile Index and Identification code.

The API 20E identification strip protocol consisted in the inoculation of an isolated colony in several biochemistry tests which were done as described next.

The oxidase test must be performed before and the result should be recorded on the result sheet as it is an integral part of the final profile (21st identification test).

The strip preparation was made as follows:

An incubation box (tray and lid) was prepared distributing 5 ml. of distilled water .The strip was removed from its packaging and placed in the incubation box.

The inoculums preparation was made as follows:

Using a pipette, a single well isolated colony was removed from an isolation plate (Figure 36). It is recommended to use young cultures (18-24 hours old). It was emulsified to achieve a homogeneous bacterial suspension with 5 ml of sterile distilled water placed in a sterile flask. The suspension was used immediately after preparation.

Figure 36. Removing an isolated colony to make a bacterial suspension to inoculate the API 20E strip.

The inoculation of the strip was made as follows:

Using the same pipette, tube and cupule of the tests CIT , VP and GEL were filled with the bacterial suspension. Anaerobiosis was created by overlaying with mineral oil in the tests ADH, LDC, ODC, H2S and URE. The incubation box was closed and incubated at 36°C. for 18-24 hours.

After the incubation period, the strip was read by referring to the Reading table (Included in the API 20E test kit). If three or more tests (GLU test (+) or (–)) where positive, all the spontaneous reactions were recorded on the result sheet and then the tests which require the addition of reagents were revealed:

TDA Test: one drop of TDA reagent was added. A reddish brown color indicates a positive reaction recorded on the result sheet.

IND Test. one drop of JAMES reagent was added. If a pink color developed in the whole cupule indicates a positive reaction recorded on the result sheet.

VP Test. one drop each of VP 1 and VP 2 reagents was added. If after 10 minutes turn into a pink or red color it indicates a positive reaction recorded on the result sheet but if a slightly pink color appeared after 10 minutes, the reaction was considered negative.

An important consideration is that the indole production test was performed last since this reaction releases gaseous products which interfere with the interpretation of other tests on the strip. If the number of positive tests (including the GLU test) before adding the reagents was less than 3 the strip was reincubated for 24 more hours without adding any reagents and then reveal the tests requiring the addition of reagents (as mentioned before).

The strip interpretation was done as follows:

On the result sheet, the tests are separated into groups of 3 and a value 1, 2 or 4 is indicated for each. By adding together the values corresponding to positive reactions within each group, a 7-digit profile number is obtained for the 20 tests of the API 20 E strip. The oxidase reaction constitutes the 21st test and has a value of 4 if it is positive.

The strain identification was obtained searching the numerical profile using the Analytical Profile Index.

If the seven digit profile is not discriminatory enough, the following supplementary tests (of the same API 20E strip) were carried out:

-Reduction of nitrates to nitrites (NO_2) and N_2 gas (N_2).

-Motility (MOB).

-Growth on MacConkey agar medium (McC).

-Oxidation of glucose (OF-O).Fermentation of glucose (OF-F).

These supplementary tests, indicated in the introduction, may be used to form a nine digit profile. In this case identification is obtained using the identification software (Biomerieux Inc., France).

Data analysis. Mean and standard deviations were calculated for each parameter measured. Differences among groups were determined using one-way ANOVA and means were compared by t-student test ($P < 0.05$) using a SPSS software (SPSS 11.5 for Windows; SPSS Inc., Chicago, IL, USA).

4. Experimental developments

4. Experimental developments

4.1 Experiment One

4.1.1Objectives

Two different treatments were setup to improve the cell concentration growth of *N.gaditana* in the traditional bag culture for 10 days at the right side of ICCM´s hatchery microalgae room.

4.1.2 Materials and methods

This experiment confronted two different treatments of microalgae culture done consecutively one after the other alternating bag position to avoid any effect of the light intensity received on the bags. The first treatment consisted in the traditional bag culture inside the microalgae room, one single nutrient dose without CO_2 ($WOCO_2$) while the other treatment consisted in the same culture bags adding CO_2 gas (WCO_2) into the bags to increase the available carbon (C) in the culture water medium for photosynthesis processes and boost the algae cell density; the experiments were done for 10 days until the stationary growth phase was reached. The algae culture was developed in six 230 L traditional bags located in one side of the microalgae room (Figure 37); the six bags had 24 hours of continuous illumination due to two sets of 12 lamps each on one side of the bags. The other surfaces of the bags were exposed to the sunlight which breaks through in some parts of the algae room. Each bag was considered as a replicate of each treatment.

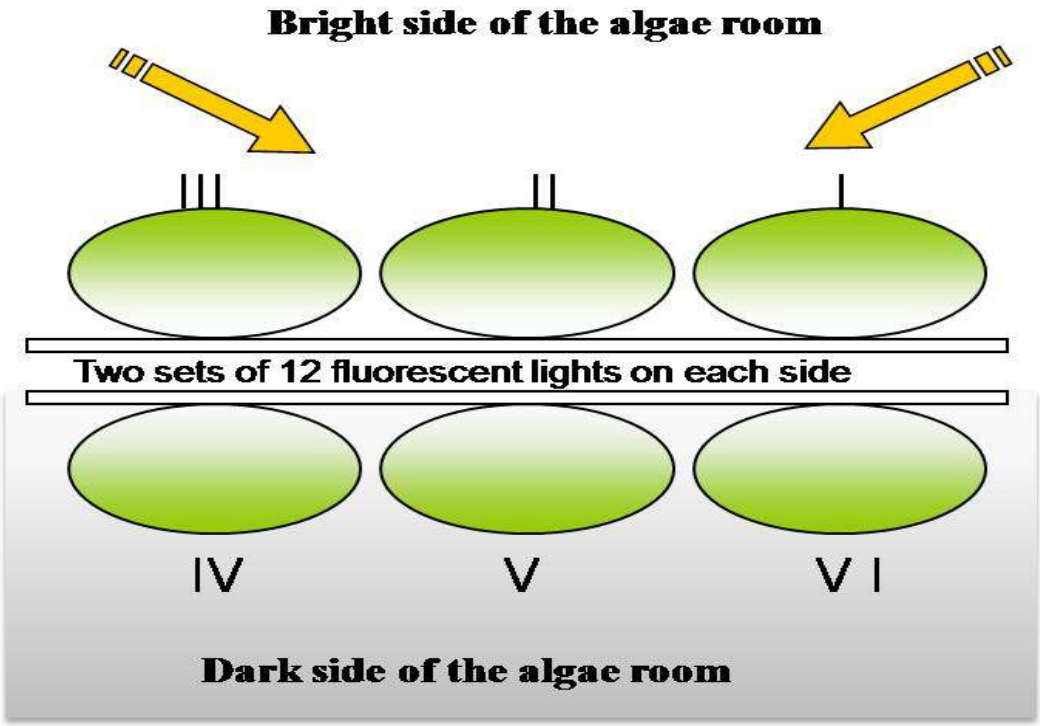


Figure 37. Right side of ICCM`s microalgae room where the first experiment took place.

In first place nutrients were added to the water at the beginning of culture, also CO_2 gas hoses were placed inside three of the six culture bags (in positions I, III and V); The other three bags (II, IV and VI) were cultured as usual (without CO_2 gas income). The CO_2 system consisted in a CO_2 gas cylinder with an electro-valve on it which was commanded by a pH controller and a pH sensor which was installed inside the bag V (Figure 38 A, B and C) The pH sensor inside the bag was continuously monitoring bag`s pH, every time pH values descend below 7.5 the pH controller opened the electro-valve on the gas cylinder and the gas flowed to the bags until pH values returned over 7.5

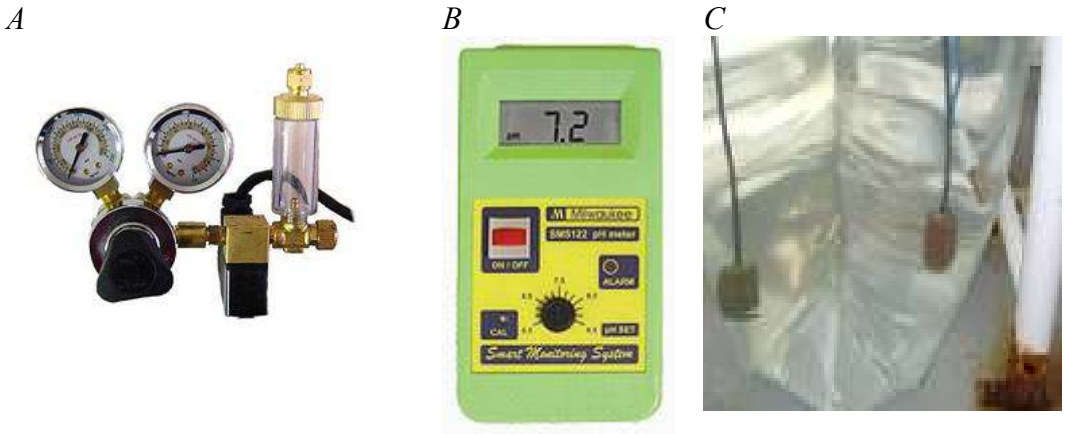


Figure 38. A. Electro-valve on top of the gas cylinder B. pH controller, it opens and closes the electro-valve depending on the pH values. C. Blue hose for CO_2, each one of the three bags of the treatment had one gas diffuser.

After this experiment was completed the six bag culture was started again changing the bag positions for CO_2 gas income (position II, IV and VI) while the bags numbered as I, II and III were cultured without CO_2 gas income. The CO_2 gas system was as mentioned before with the pH sensor inside bag number IV. Each treatment was left for 10 days to reach the exponential growth phase. Every day cell growth was evaluated and pH values were measured. One sample of each bag ($WOCO_2$ and WCO_2) was taken for biochemistry analysis on the 10^{th} day.

4.1.3 Results

After 10 days of continuous microalgae culture, no differences were observed between two treatments. The microalgae growth showed three phases, the lag phase from day 0 to 1, followed by the exponential phase from day 2 to the late exponential phase reached on the day 7 of the $WOCO_2$ treatment and on day 9 in the WCO_2 treatment.

The highest cell concentration (x 10^6 cells $(ml)^{-1}$) reached was 54.76 ± 21.22 in the cultured algae bags with CO_2 on the 9^{th} culture day, while the other treatment ($WOCO_2$) reached a highest cell concentration of 45.91 ± 15.57 on the 7^{th} culture day decreasing until the 10^{th} day was reached, very similar to the first one but the highest value was reached two

days before than the other treatment as shown in figure 39. No significant differences (P>0.05) were observed between the two treatments.

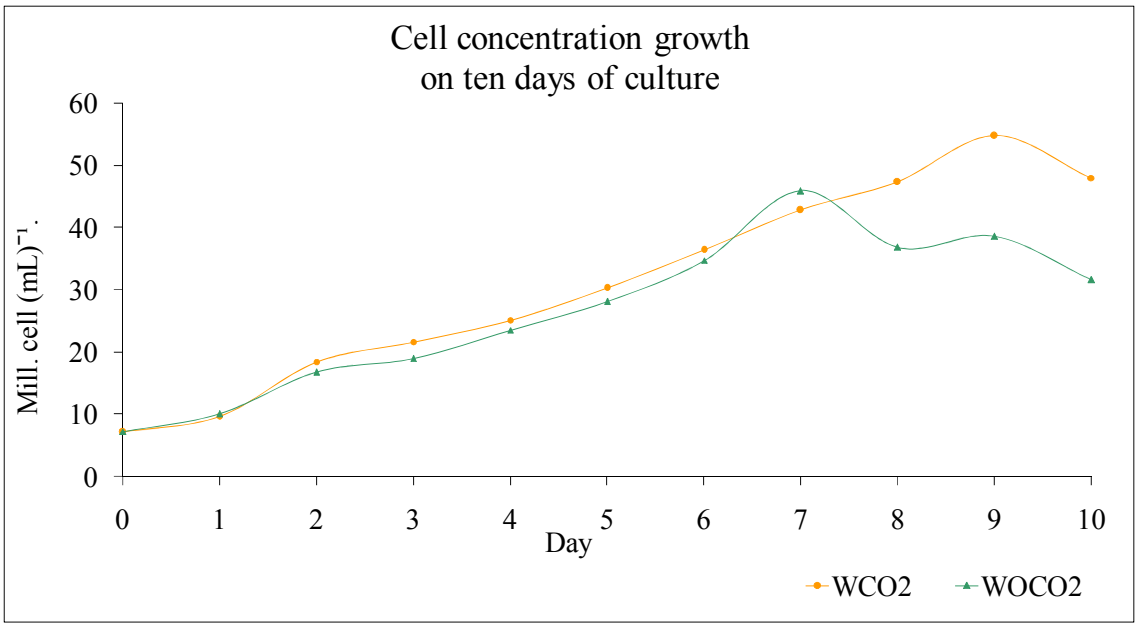


Figure 39. Effect on the cell concentration growth on *N.gaditana* culture.

Two similar growth patterns were observed with the Foto Absorbance Units (FAU) of water turbidity in both treatments, the turbidity values ascend according to the culture days (Figure 40) from 153.17 ± 16.64 on day 0 to 785.5 ± 178.1 on day 10 in the WCO_2 treatment while the $WOCO_2$ treatment began with 149.83 ± 20.43 increasing until the day 9 with 781.5 ± 110.36, and then decreased on the 10^{th} day to 768.33 ± 188. Even though the treatment with CO_2 and double nutrient dose showed turbidity values above the treatment without CO_2, no significant differences were observed during the experiment.

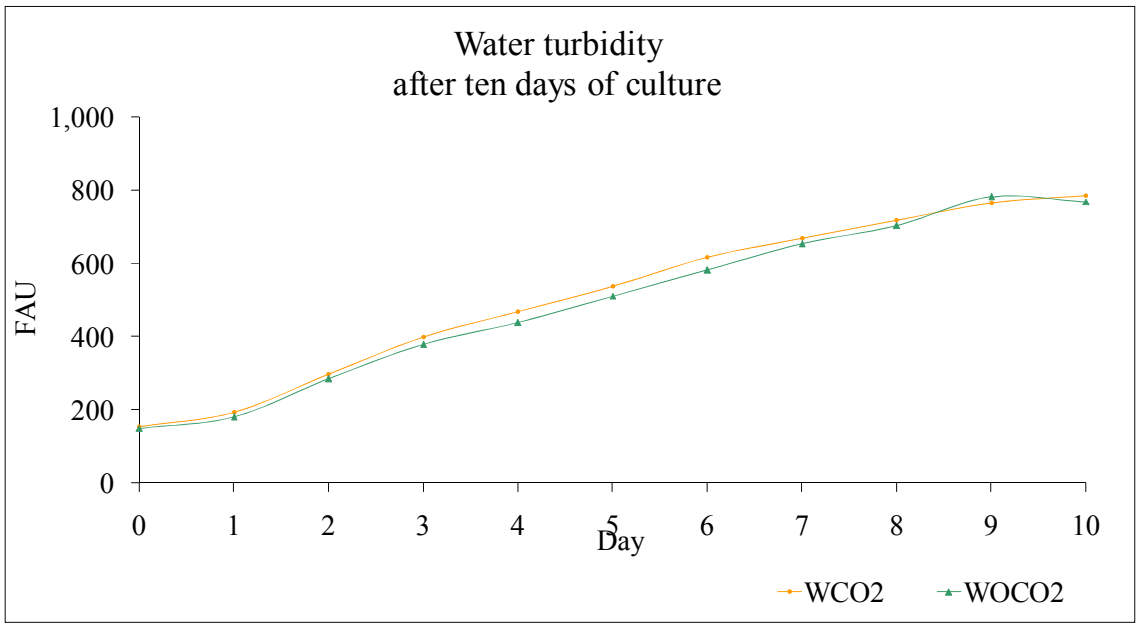


Figure 40. Water turbidity measures on both treatments

Water turbidity and cell concentration showed a high correlation between their values along the experiment in both treatments except for the last days when the treatment without CO_2 ($WOCO_2$) stopped growing in an exponential way. The treatment with CO_2 (WCO_2) had correlated values of R^2=0.9716 while the treatment without CO_2 had a correlated value of R^2=0.8469 due to the lost of cell concentration in the last two days as shown in figure 41.

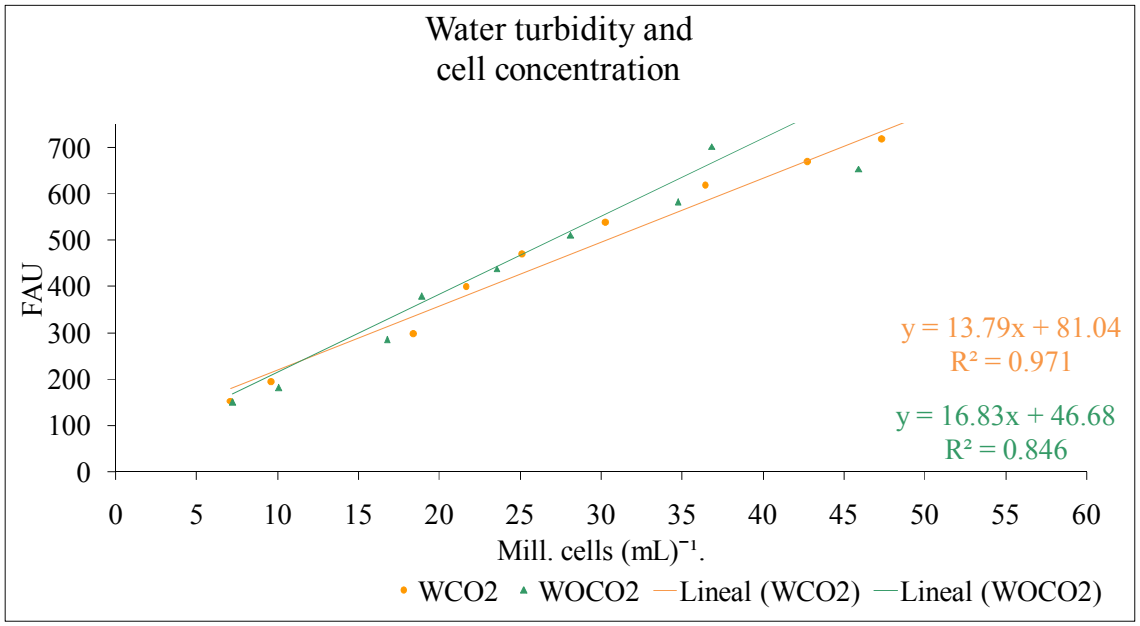


Figure 41. Correlation between water turbidity and cell concentration in both treatments.

After ten days of microalgae culture the biomass production g $(L)^{-1}$ showed no significant differences between the two treatments (Figure 42). The microalgae production (g $(L)^{-1}$) was 0.36 ±0.01 on the treatment with CO_2 and 0.37 ± 0.08 on the treatment without CO_2. There were no significant differences between each other.

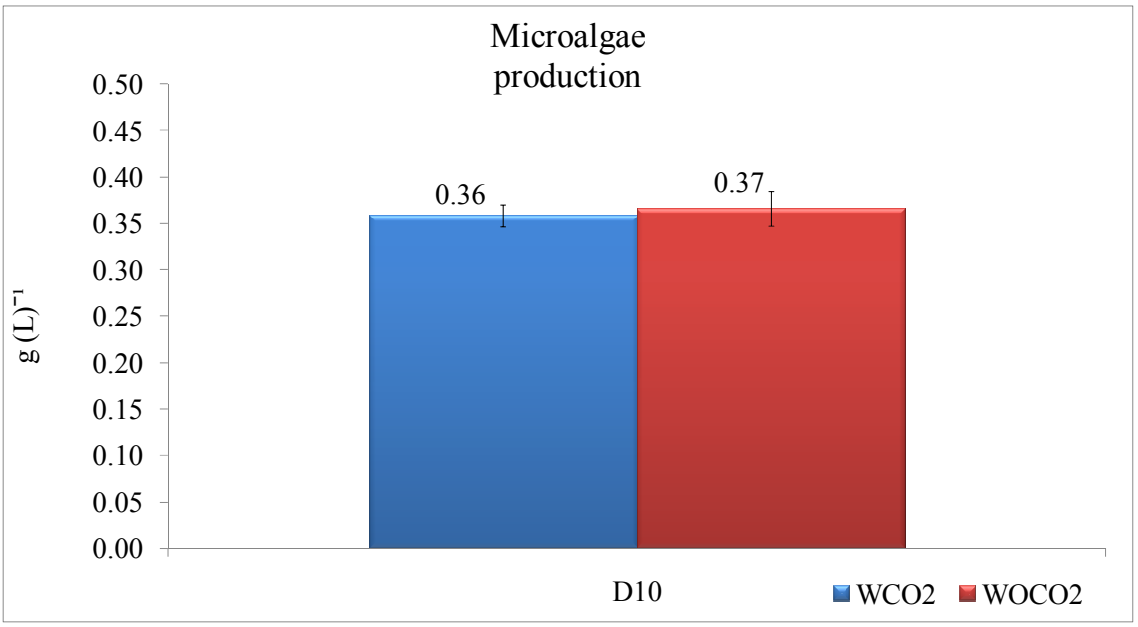


Figure 42. Microalgae production on both treatments after ten days of growth.

The pH values in both treatments were similar but the pH values were more stable around 8.4 ±0.03 in the microalgae culture bags with CO_2, as shown on figure 43. While in the ones without CO_2 pH increased above 9 ±0.02 in the first three days and maintained values over 8.8 during the rest of the culture. The treatment without CO_2 showed more instability and higher values than the treatment with CO_2.

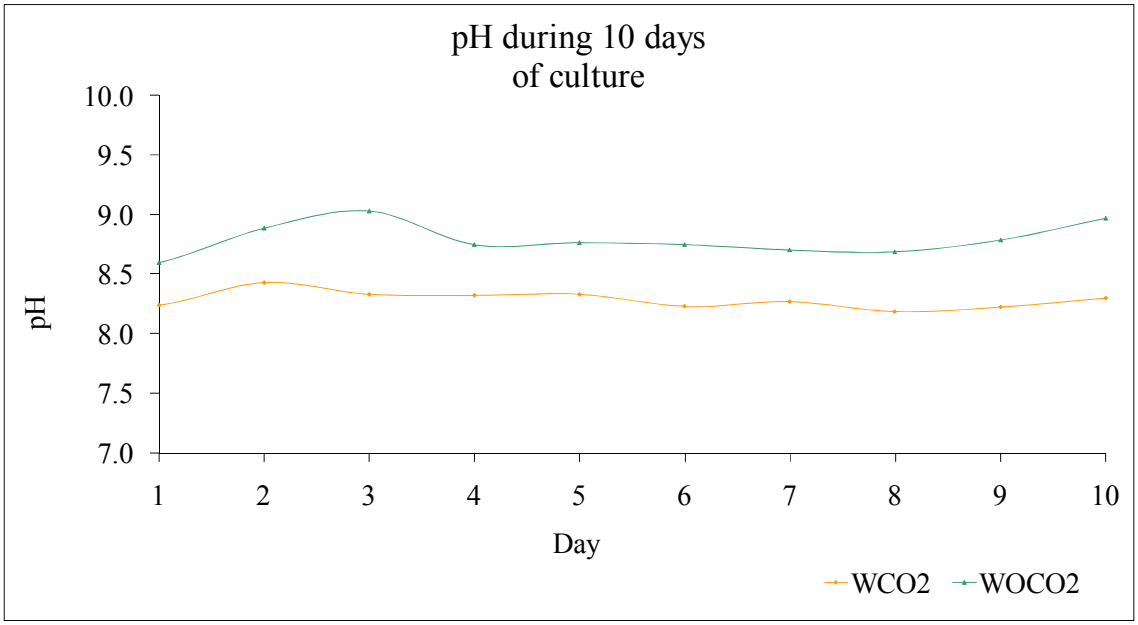


Figure 43. Water culture daily pH values.

4.1.4 Biochemistry analysis results

The biochemical analysis shown on figure 44 corresponds only to the microalgae composition at the 10th culture day. There were no significant differences observed in the proximal analysis. The microalgae samples moisture (%) content were 79.6 ± 0.33 on the microalgae treatment with CO_2 and 79.3 ± 3.24 on the treatment without CO_2. The protein content of the bag with CO_2 was 61.5, and 60.6 in the treatment without CO_2. Total lipids (%DW) observed were 10.21 ±3.56 on the treatment with CO_2 and 9.46 ±1.27 on the treatment without CO_2. The ash content was a little smaller in the treatment with CO_2 with 6.4 than the treatment without CO_2 with 5.7; at last the carbohydrate content was 22.0 in the treatment with CO_2 and 23.9 in the treatment without CO_2.

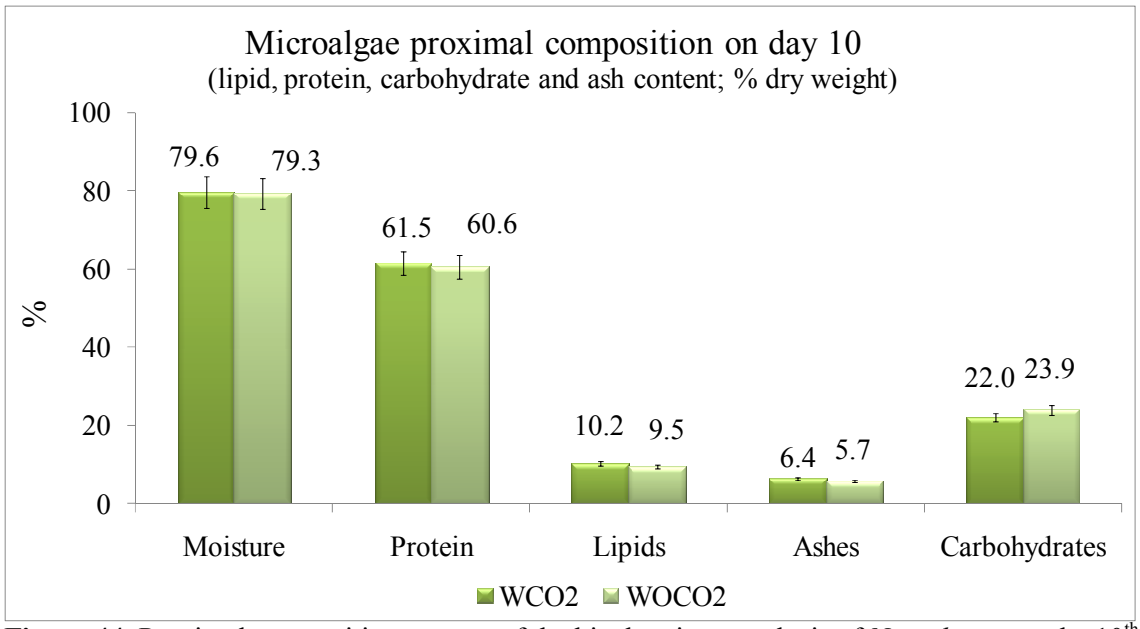


Figure 44. Proximal composition content of the biochemistry analysis of *N. gaditana* on the 10th culture day.

The microalgae fatty acids composition of *N. gaditana* values (%DW) presented in table IV showed a higher content of saturated fatty acids in the treatment without CO_2 which was 2.196 ±0.321 than in the treatment without CO_2 with 1.953 ±0.673, also the mono-unsaturated fatty acids where higher in the same way with 3.023 ± 0.225 in the treatment with CO_2 and 2.504 ± 0.348 in the other one. Oleic acid was almost equal in both samples with 0.525 ± 0.098 in the treatment with CO_2 and 0.5 ± 0.030 in the treatment without CO_2. The n-3 HUFA`s, ARA (arachidonic acid), EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid) are presented in figure 45.

The small DHA/EPA proportion in both treatments responds to the little DHA content found in both samples in contrast with the high EPA content; however this proportion was higher in the treatment with CO_2 with 0.078 ± 0.026, while the other treatment was 0.003 ± 0.001.The high proportion between fatty acids n-3 and n-6 also responds to the high EPA content in both samples, it was 5.567 ± 2.213 in the treatment with CO_2 and 4.884 ± 1.973 in the other one.

Table IV. Fatty acid composition (% dry weight) of the microalgae samples of each treatment

Fatty Acids	WCO_2		$WOCO_2$	
∑ **Saturated**[9]	1.953	± 0.673	2.196	± 0.321
∑ **Mono-unsaturated**[2]	2.504	± 0.348	3.023	± 0.225
∑ **n-3**[3]	3.176	± 0.540	3.361	± 0.636
∑ **n-6**[4]	0.570	± 0.487	0.688	± 0.160
∑ **n-9**[5]	0.577	± 0.455	0.538	± 0.038
∑ **n-3HUFA**[6]	2.983	± 0.388	3.263	± 0.628
14:00	0.675	± 0.634	0.703	± 0.037
16:00	1.157	± 0.982	1.402	± 0.267
16:1 n-7	1.694	± 0.105	1.910	± 0.088
18:00	0.048	± 0.013	0.024	± 0.002
18:1 n-9	0.525	± 0.098	0.500	± 0.030
18:1 n-7	0.062	± 0.024	0.500	± 0.030
18:2 n-6	0.157	± 0.031	0.155	± 0.034
18:3 n-3	0.025	± 0.000	0.018	± 0.002
20:1 n-9	0.024	± 0.025	0.006	± 0.002
ARA	0.346	± 0.019	0.481	± 0.014
EPA	2.691	± 0.229	3.217	± 0.617
DHA	0.210	± 0.056	0.008	± 0.002
DPA (22:5n-6)	0.004	± 0.001	0.000	± 0.000
DHA/22:5 n-6	79.288	± 8.148	0.000	± 0.000
EPA/ARA	7.782	± 1.366	6.688	± 1.389
ARA/EPA	0.129	± 0.017	0.150	± 0. 016
DHA/EPA	0.078	± 0.026	0.003	± 0.001
DHA/ARA	0.608	± 0.102	0.017	± 0.008
oleic/DHA	2.498	± 0.556	61.191	± 4.474
oleic/n-3HUFA	0.176	± 0.066	0.153	± 0.049
n-3/n-6	5.567	± 0.213	4.884	± 0.973

[9] (1) Includes 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 24:0; (2) Includes 14:1n-5, 14:1n-7, 16:1n-9, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-11, 22:1n-9 and 22:1n-7; (3) Includes 16:2n-3, 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3; (4) Includes 16:2n-6, 18:2n-6, 18:3n-6,18:4n-6, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-6, 22:3n-6, 22:4n-6 and 22:5n-6; (5) Includes 16:1n-9, 18:1n-9, 18:2n-9, 18:3n-9, 20:1n-9,20:2n-9, 20:3n-9 and 22:1n-9; (6) Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3.

Total n-3 HUFA`s (%DW) found in both treatments were 2.98 ± 2.388 (WCO_2) and 3.263 ± 0.628 ($WOCO_2$) as shown in figure 45. There were significant differences (P>0.05) observed between the two treatments. The highest (n-3) fatty acid value corresponds to the EPA with 2.69 ± 2.029 (WCO_2) and 3.217 ± 0.617 ($WOCO_2$), followed by ARA with 0.35 ± 0.319 and 0.481 ± 0.114 and DHA at last with 0.21 ± 0.25 and 0.008 ± 0.002, as shown on figure 45.

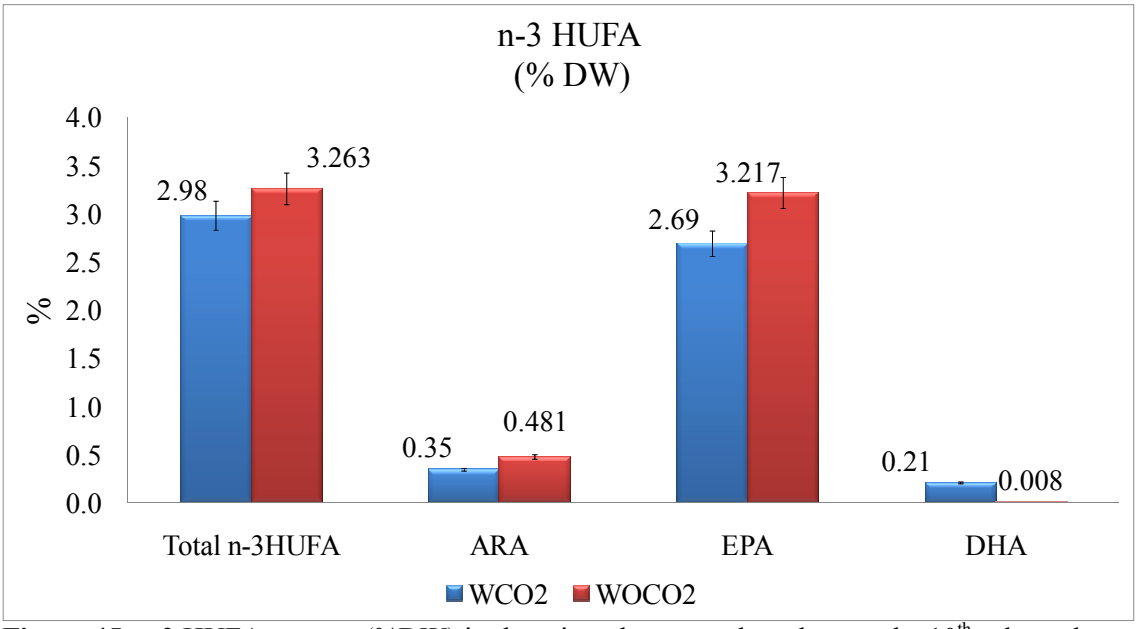


Figure 45. n-3 HUFA content (%DW) in the microalgae samples taken on the 10th culture day.

4.2 Experiment two

4.2.1 Objectives

Two different treatments were setup to improve the cell concentration growth of *N.gaditana* in the traditional bag culture for 20 days in both sides of the ICCM´s hatchery microalgae room and obtain the best dilution rate for daily partial harvest.

4.2.2 Material and methods

As a result of the previous experiment where the cell concentration growth was not improved by the addition of CO_2 gas, in this experiment both sections of the microalgae room composed by 12 bags of 230 L each (Figure 46) were used at the same time to evaluate *N. gaditana* production in 20 days to see the effect of adding double nutrient dose plus CO_2 gas income in the cell concentration growth up to the stationary phase. After the 10^{th} culture day, daily dilutions began, collecting 10%, 15% and 20% volume of three pairs of bags **(**with opposite position in the microalgae room between each other) and replacing them with the same volume of enriched water for 10 more days to evaluate the cell concentration recovery after the dilution as shown in table V.

Table V. Dilution rate of bags in both treatments

Treatment	20%	15%	10%
Bags WCO_2	V & VI	I & II	III & IV
Bags $WOCO_2$	XI & XII	IX & X	VII & VIII

The twelve bags were started with twice the nutrient dose at the beginning of the experiment with an initial concentration ($x10^6$ cells $(ml)^{-1}$) of 4.49 ± 0.081.

The first treatment (WCO_2) was developed at the right side of the microalgae room where six bags (I – VI) were traditionally cultured but equipped with a CO_2 gas dispenser to all bags and a pH controller installed in bag IV. The second treatment ($WOCO_2$) was made at

the left side of the microalgae room where six 230 L bags (VII – XII) were traditionally cultured without using CO_2 as shown in figure 46.

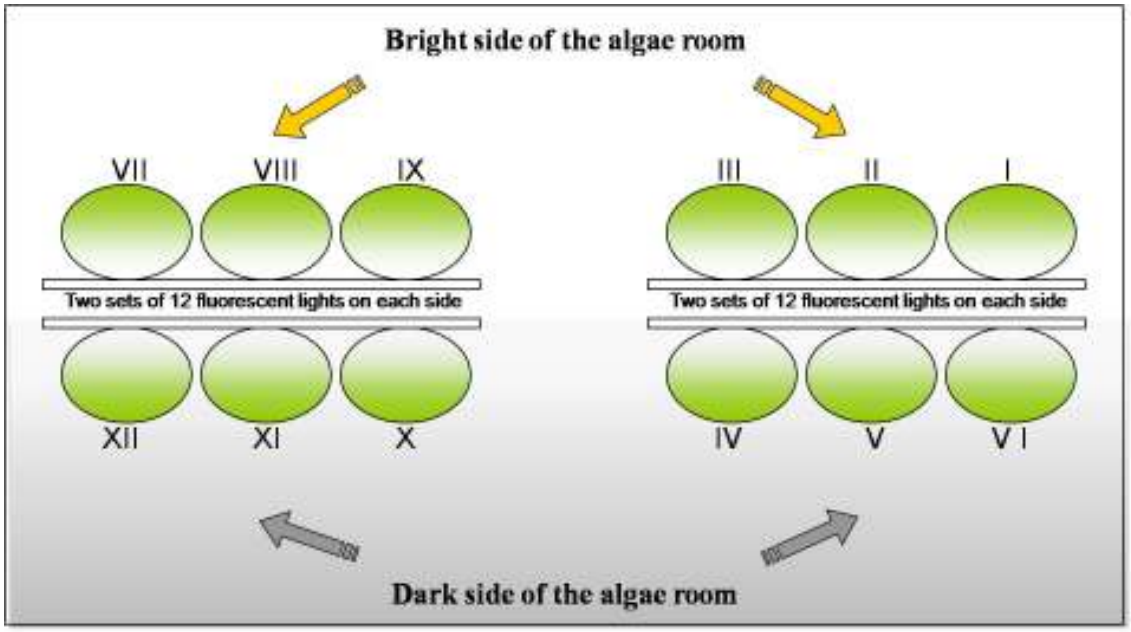


Figure 46. Microalgae room diagram shows the experiment bags that were used for the experiment.

Every day cell growth concentration was evaluated and water culture parameters measured on every bag and averaged for the data compound. One sample of each bag was taken for biochemistry analysis on the 3rd, 10th, and 20th day as it is described in materials and methods.

4.2.3 Results

After the first ten days of microalgae culture **(**Figure 47**)**, no significant differences were observed between the cell concentration growths of the two treatments. The microalgae growth showed three phases, the lag phase from day 0 to 2, followed by the exponential phase from day 3 up to the late exponential phase reached on the day 9 on the CO_2 treatment bags and on day 10 in the treatment without CO_2.

The highest cell concentration (x10^6 cells $(ml)^{-1}$) reached was 45.916 ± 10.869 on the 9th culture day in the first treatment (WCO_2) reaching the exponential phase, while the other

treatment ($WOCO_2$) reached a cell concentration of 41.375 ± 10.93 on the 10th culture day as shown in figure 47. No significant differences were observed between the two treatments.

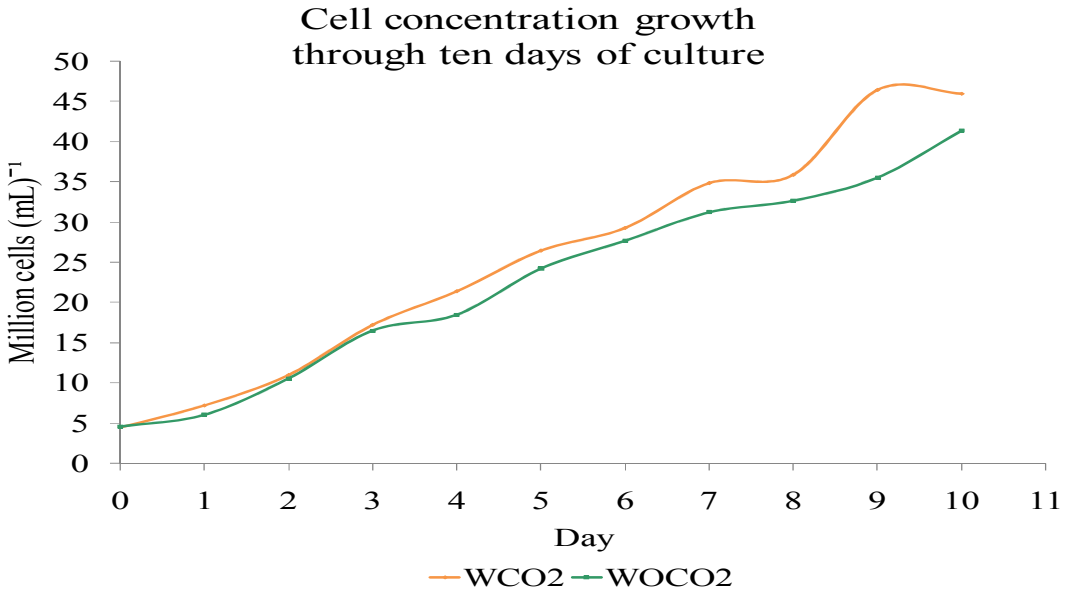


Figure 47. Growth of *N. gaditana* in both treatments during the first ten days of experiment.

Two similar growth patterns were observed with the FAU of water turbidity in both treatments; the turbidity values ascend according to the culture days due to the cell concentration (Figure 48) from 60 ± 0 in both treatments on day 0 up to722 ± 134 in the treatment with CO_2 and 709 ± 164 on the treatment without CO_2 on day 10 when the late exponential phase was reached. No significant differences were observed between the two treatments.

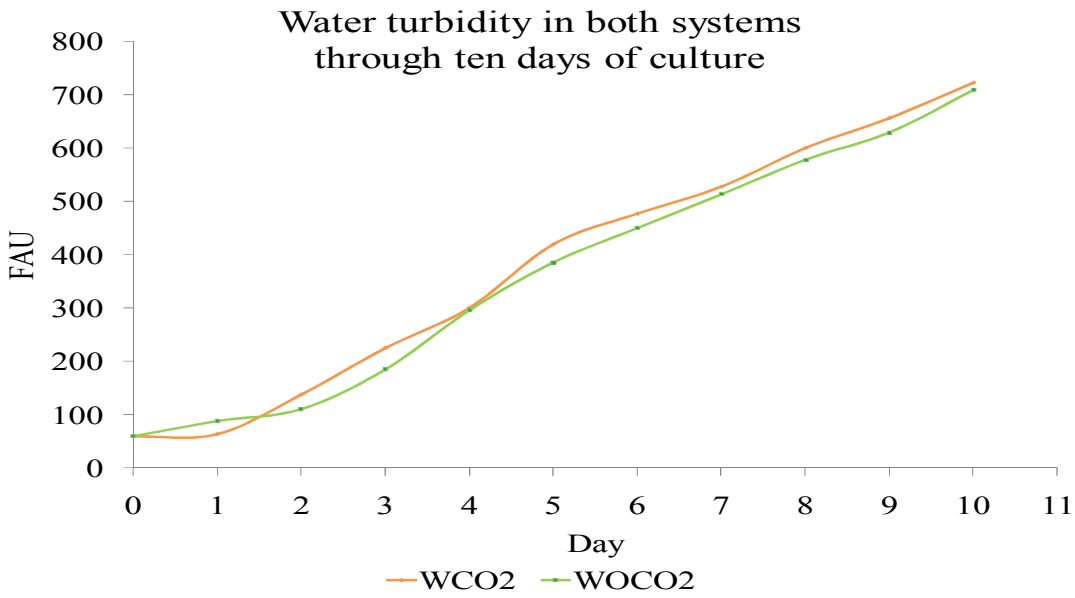


Figure 48. Water turbidity measures on both treatments during the first 10 culture days.

Water turbidity and cell concentration showed (Figure 49) a high correlation between their values along the experiment in both treatments. The treatment with CO_2 had correlated values of R^2=0.982 while the treatment without CO_2 had a correlated value of R^2=0.983 This correlation was reached through the lag phase from day 0 to 2, exponential growth phase from day 3 up to the late exponential phase reached on day 10 (Figure 49).

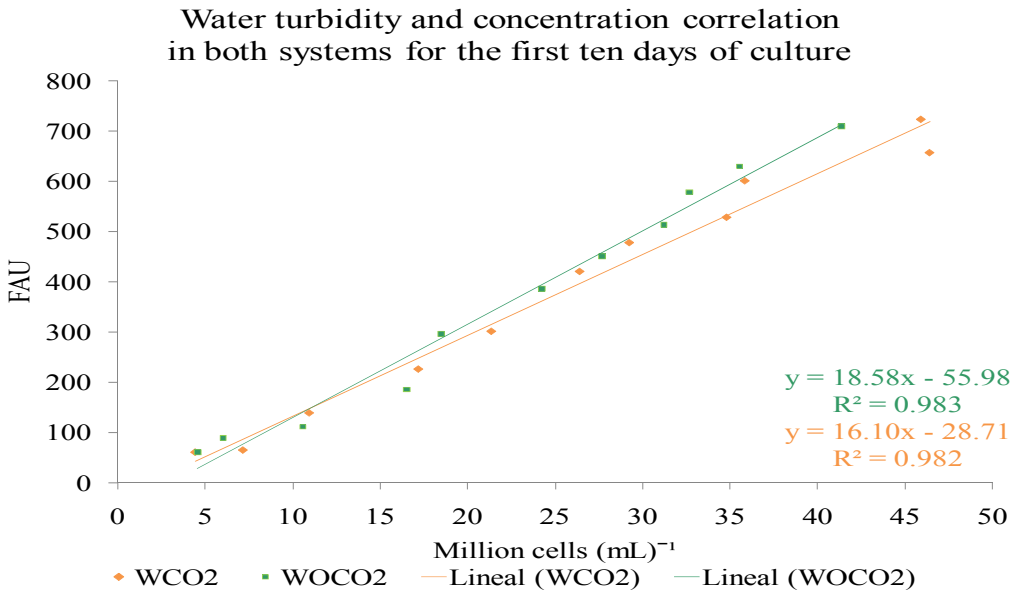


Figure 49. Correlation between water turbidity and cell concenctration in both treatments for the first ten days of the experiment.

There were no significant differences in the microalgae production between the two treatments. The biomass production average from culture day 10 to 30 was 0.31 ± 0.077 in the treatment with CO_2, while the average biomass production in the treatment without CO_2 was 0.303 ± 0.071 on the same culture days. The highest biomass production was reached by the two treatments on day 10, with 0.475 (WCO_2) and 0.428 ($WOCO_2$). In contrast the lowest rate production was obtained on the 20th culture day in the treatment with CO_2 with 0.248 $g(L)^{-1}$ against 0.221 $g(L)^{-1}$ in the treatment without CO_2 (Figure 50). No significant differences were observed between two treatments.

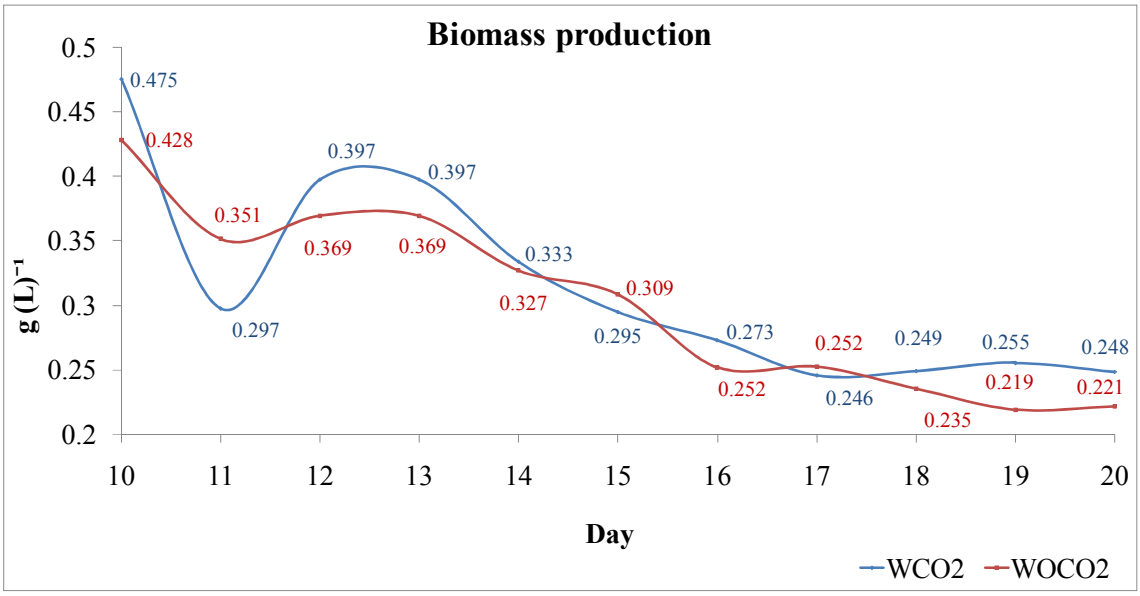


Figure 50. Microalgae production during daily 10% harvest.

The pH values in both treatments were similar but the pH values were more stable around 7.54 ± 0.05 in the microalgae culture bags with CO_2 during the first ten days of culture, while in the ones without CO_2 pH maintained values over 7.65 ± 0.206 with some oscillations on day 3 and day 8 of culture. The treatment without CO_2 showed more instability and higher values than the treatment with CO2 in the first ten days of culture (Figure 51).

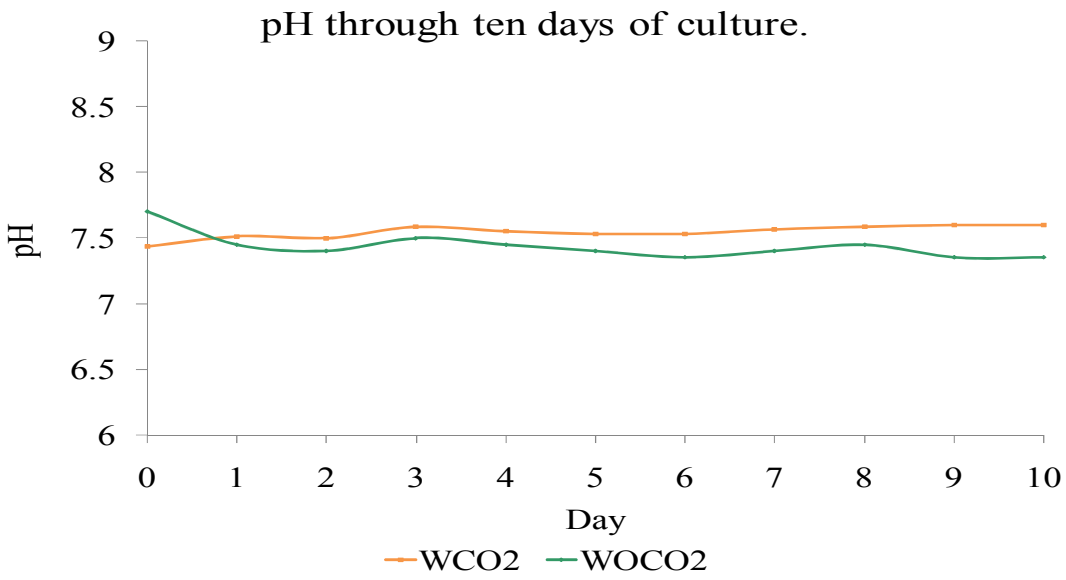


Figure 51. Water culture daily pH values.

The light inside the microalgae room is never constant because the sunlight changes position through the day and the intensity entering the room is blocked by a semi-transparent mesh which not aloud an intensity above 5000 lux in the bags facing front the mesh. The bags that do not face the mesh in the dark side of the room (Figure 46) had lower intensity less than 500 lux; this big difference between light intensity in the surface of the bags facing out (the mesh) and the bags facing the wall cause big standard deviation between them as is showed on figure 52.

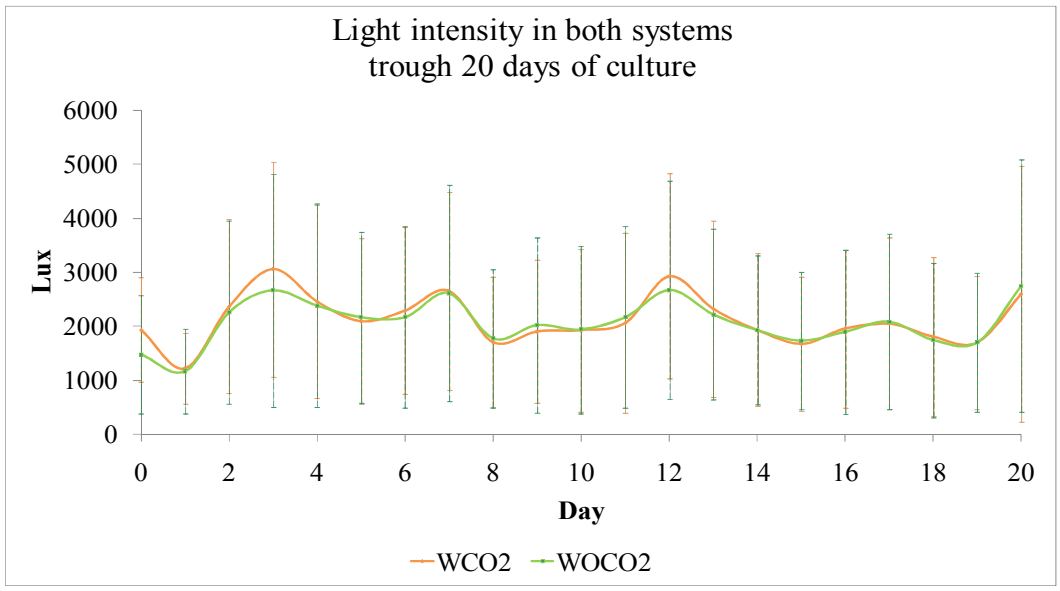


Figure 52. Light intensity in both treatments through 20 days of experiment.

4.2.3.1 Results after harvest

After the 10th culture day 10% of the volume of the bags III & IV (WCO_2) and bags VII & VIII ($WOCO_2$) were daily harvested and replaced with the same volume of enriched water. The treatment with CO_2 decreased it cell concentration after the first dilution from 45.916 ± 10.869 on the day 10 to 28.76 ± 9.072 on the day 11, recovering up to 38.427 ± 14.431 on the day 13 and decreasing again down to 24.01 ± 13.136 on the 20th culture day. The same pattern occurred to the treatment without CO_2; in this case the first dilution took down the cell concentration from 41.375 ± 10.93 on the 10th day to 40.3 ± 11.48 on the day 11, recovering up to 43.593 ± 15.159 on the day 13 and then decreasing to 28.2 ± 9.68 on the 20th culture day (Figure 53). The cell concentration showed significant differences (P>0.05) between treatments. The highest cell concentration was 35.1 ± 6.09 in the treatment without CO_2 compared with the cell concentration average of the treatment with CO_2 which was 28.3 ± 8.06 during 10% daily dilutions.

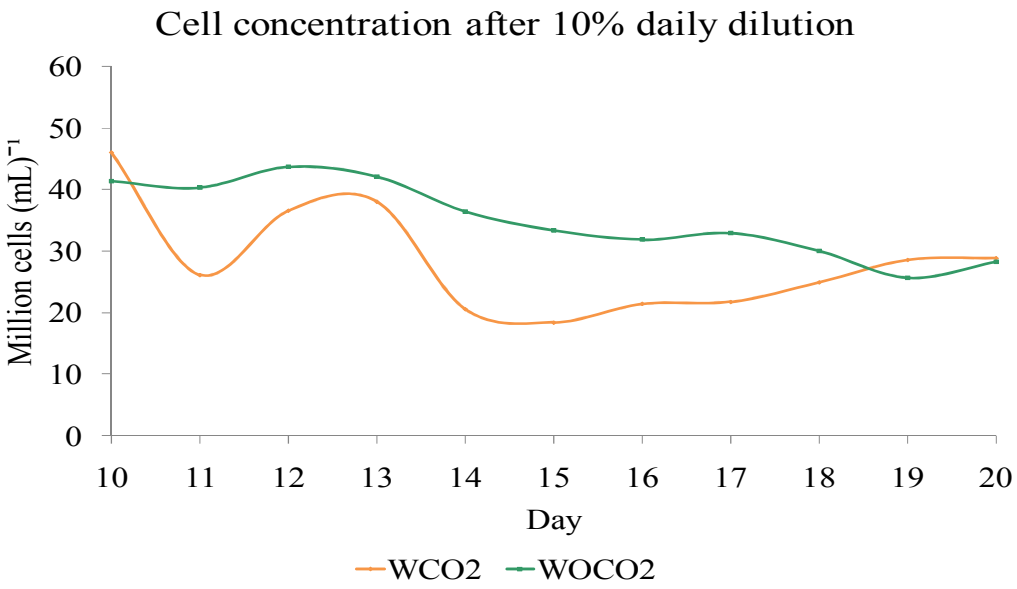


Figure 53. Cell concentration after 10% daily dilution.

Two similar growth patterns were observed with FAU of water turbidity in both treatments; the turbidity values descended according to the culture days due to the cell concentration (Figure 54) from 709 ± 164 on day 10 down to 473 ± 149 on day 20 in the

treatment without CO_2. The treatment with CO_2 showed an oscillating pattern of cell concentration (turbidity absorbance) recovery from the day 10 with 722 ± 134 to 815 ± 146 on day 13 followed by an abrupt water turbidity decrease down to 436 ± 430 on the 14th day remaining stable until the day 20 was reached with an absorbance of 380 ± 311 in the treatment with CO_2. The same significant differences found in the cell concentration growth were observed between the two treatments in the water turbidity absorbance (Figure 54).

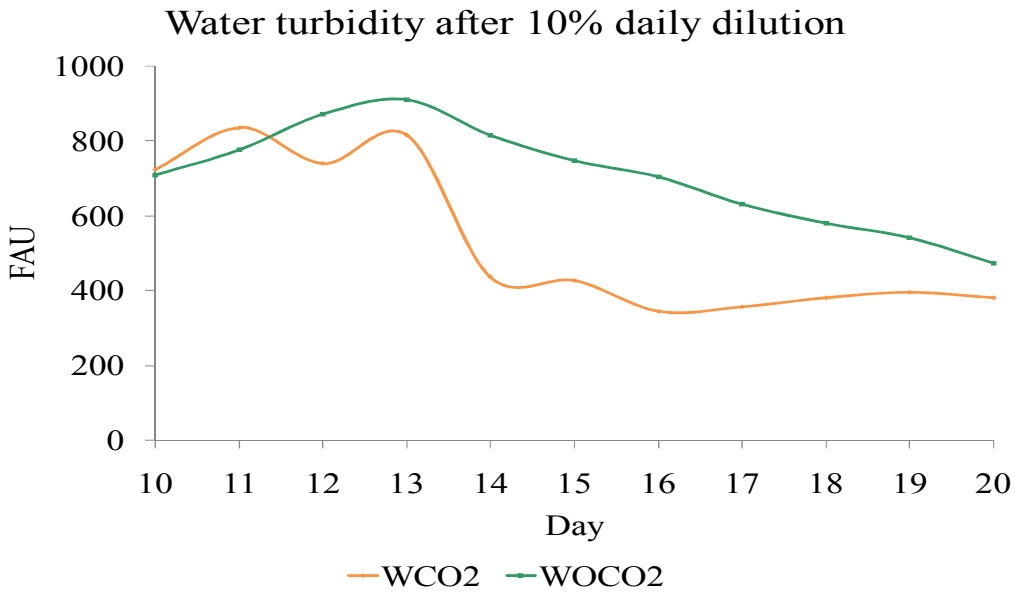


Figure 54. Water turbidity measures on both treatments after 10% daily dilution.

Water turbidity and cell concentration showed a low correlation between their values along the last 10 days of the experiment in the treatment with CO_2 with a correlated value of R^2=0.687 while the treatment without CO_2 had a higher correlated value of R^2=0.765 This correlation was reached through the stationary phase from day 10 to 20 as shown in figure 55.

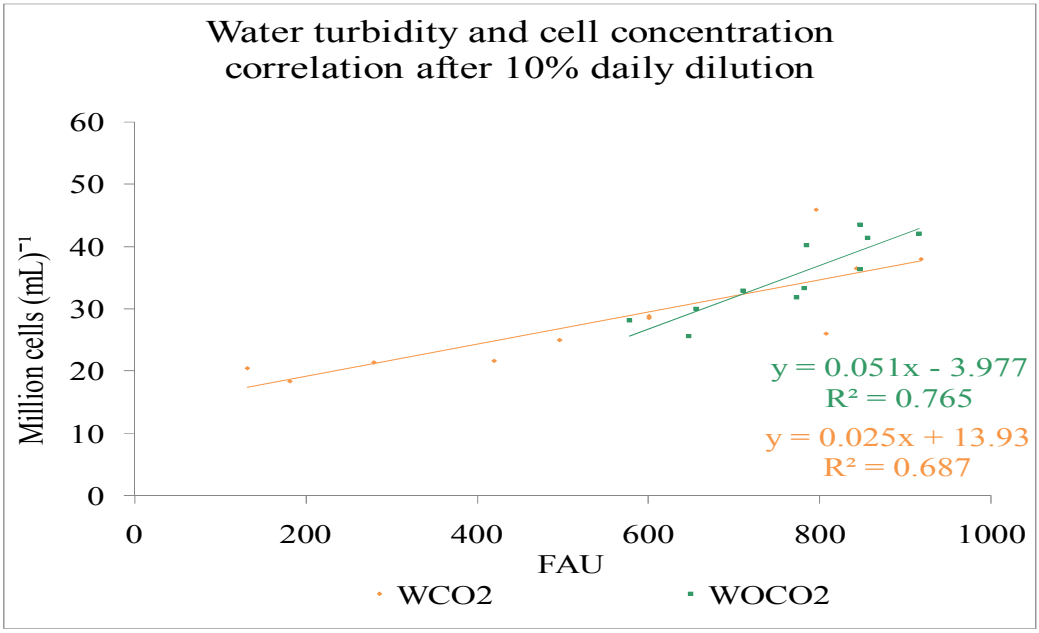


Figure 55. Correlation between water turbidity and cell concentration after 10% daily dilution in experiment.

The pH values after 10% daily dilutions in both treatments were similar but the pH values were more stable around 7.55 ± 0.092 in the microalgae culture bags with CO_2 during the last ten days of culture, while in the ones without CO_2 pH showed values over 7.68 ± 0.168 but these values increased above 8 in the last 3 culture days. (Figure 56).

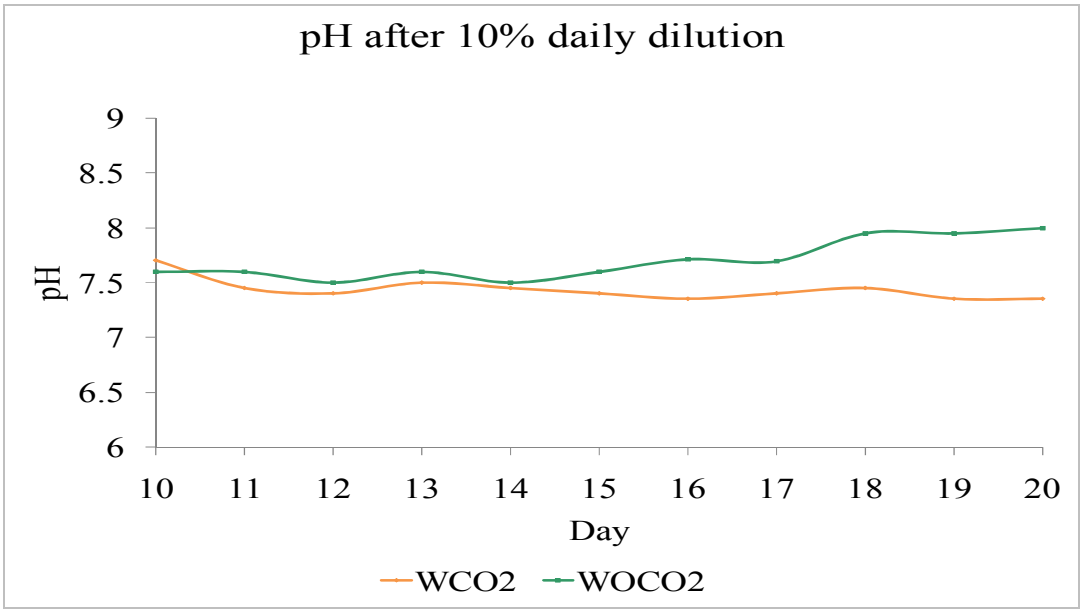


Figure 56. Water culture daily pH values.

After the 10th culture day 15% of the volume of the bags I & II (WCO_2) and bags IX & X ($WOCO_2$) were daily harvested and replaced with the same volume of enriched water.

The treatment with CO_2 decreased its cell concentration ($x10^6$ cells $(ml)^{-1}$) after the first dilution from 45.916 ± 10.869 on the day 10 to 37.63 ± 7.5 on the day 11, recovering up to 53.25 ± 13 on the day 12 and decreasing again down to 28.7 ± 11.0 on the 20th culture day.

The same pattern occurred to the treatment without CO_2; in this case the first dilution took down the cell concentration from 41.375 ± 10.93 on the 10th day to 27.3 ± 4.3 on the day 11, recovering up to 30.2 ± 5.3 on the day 13 and then decreasing to 21.1 ± 15.60 on the 20th culture day as shown in figure 57. The cell concentration growth average after the 15% daily harvest in the treatment with CO_2 was 40.74 ± 9.30 compared with the cell concentration average in the treatment without CO_2 which was 26.72 ± 5.71. There were significant differences (P>0.05) found between them.

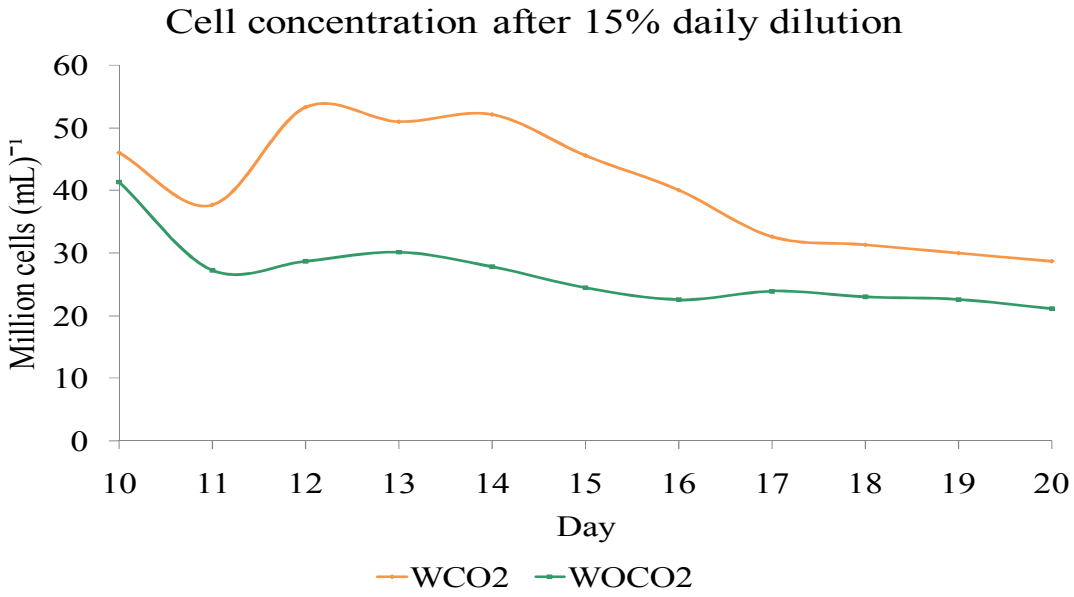


Figure 57. Cell concentration growth of *N. gaditana* on both treatments after 15% daily dilution.

Two similar growth patterns were observed with the FAU of water turbidity in both treatments; the turbidity values gradually descended according to the culture days due to the cell concentration (Figure 58) from 709 ± 164 on day 10 down to 351.5 ± 286.4 on day 20 in the treatment without CO_2. The cell concentration in the treatment with CO_2 fall from 722 ±

134 on the day 10 to 754 ±194 on day 11, recovering its cell concentration (turbidity absorbance) on day 13 to 808 ± 154, gradually decreasing until the day 20 with an absorbance of 345 ± 65.1 in the treatment with CO_2. As it happened in cell concentration growth, there were significant differences (P>0.05) found between the two treatments.

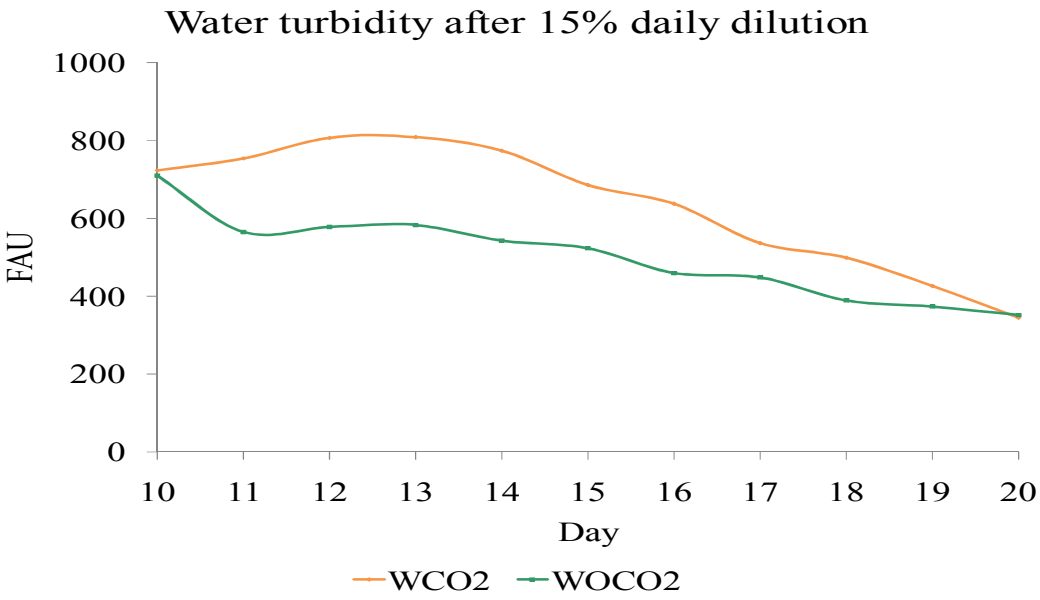


Figure 58. Water turbidity after 15% of daily dilutions.

As the cell concentration gradually decreased on both treatments, the correlation between cell concentration and water turbidity in the treatment without CO_2 was R^2=0.893 higher than the treatment with CO_2 with a correlated value of R^2= 0.454 due to its cell concentration recovery on the 12th and 13th day.

Two similar growth patterns were observed with the FAU of water turbidity in both treatments; the turbidity values gradually descended according to the culture days due to the cell concentration (Figure 59) from 624 ± 79.2 on day 10 down to 351.5 ± 286.4 on day 20 in the treatment without CO_2. The cell concentration in the treatment with CO_2 fall from 866 ± 44.5 on the day 10 to 754 ± 194 on day 11, recovering its cell concentration (turbidity absorbance) on day 13 to 808 ± 154 ,gradually decreasing until the day 20 with an absorbance of 345 ± 65.1 in the treatment with CO_2. The treatment without CO_2 was higher than the treatment CO_2.

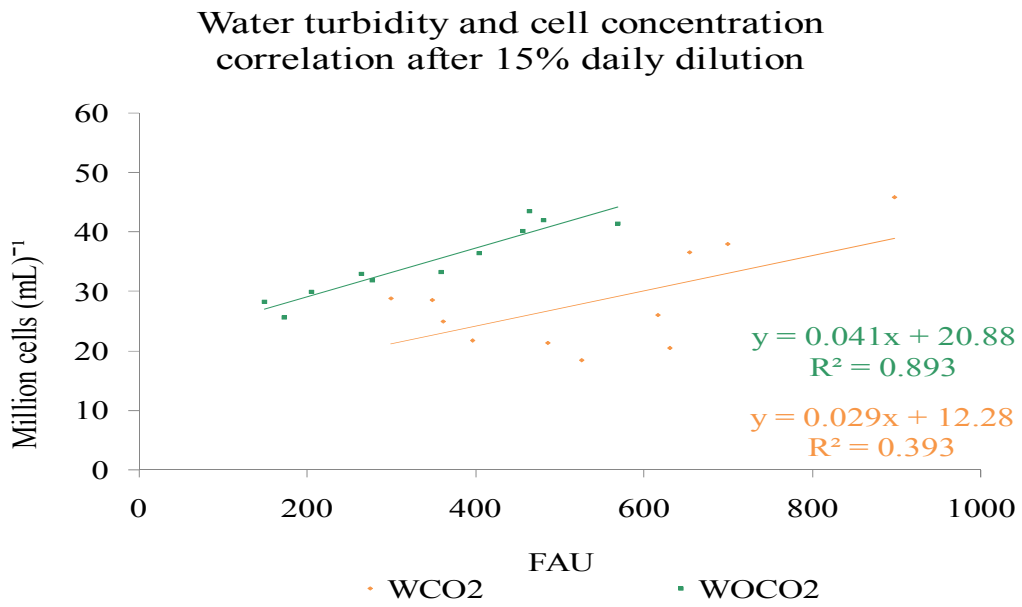


Figure 59. Correlation between both treatments after 15% daily dilutions from day 10 to 20.

The pH values in both treatments after 15% daily dilutions were similar but the pH values were more stable around 7.57 ± 0.1 in the microalgae culture bags with CO_2 during the last ten days of culture, while in the ones without CO_2 pH showed values over 7.69 ± 0.16 but these values increased above 8 in the last 3 culture days in the treatment without CO_2 , while the treatment with CO_2 kept pH values stable around 7.5 at the day 20 (Figure 60).

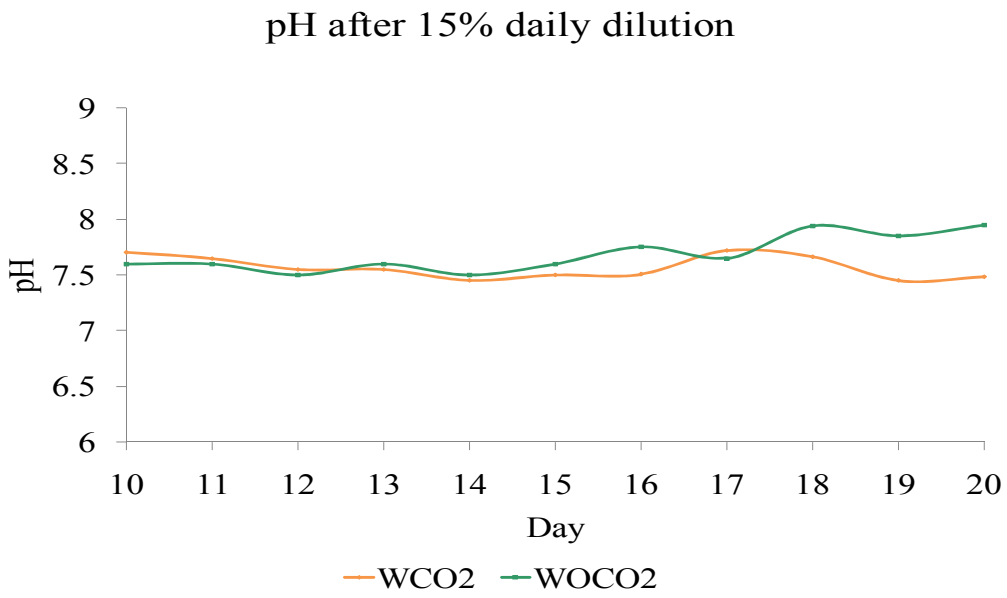


Figure 60. The pH values after 15 % daily dilutions on both treatments.

After the 10th culture day 20% of the volume of the bags V & VI (WCO_2) and bags XI & XII ($WOCO_2$) were daily harvested and replaced with the same volume of enriched water.

The treatment with CO_2 decreased its cell concentration after the first dilution from 45.916 ± 10.869 on the day 10 to 30.6 ± 19 on the day 14, recovering up to 31.8 ± 23 on the day 15 and decreasing again down to 14.9 ± 9.1 on the 20th culture day.

The same behavior occurred to the treatment without CO_2; in this case the first dilution took down the cell concentration from 41.375 ± 10.93 on the 10th day to 22.56 ± 10.2 on the day 11, recovering up to 26.28 ± 4.29 on the day 13 and then decreasing to 14.5 ± 3.01 on the 20th culture day as shown in figure 61. Besides there was a higher cell concentration in the treatment without CO_2 from culture day 10 to culture day 15, there were no significant differences found between the two treatments.

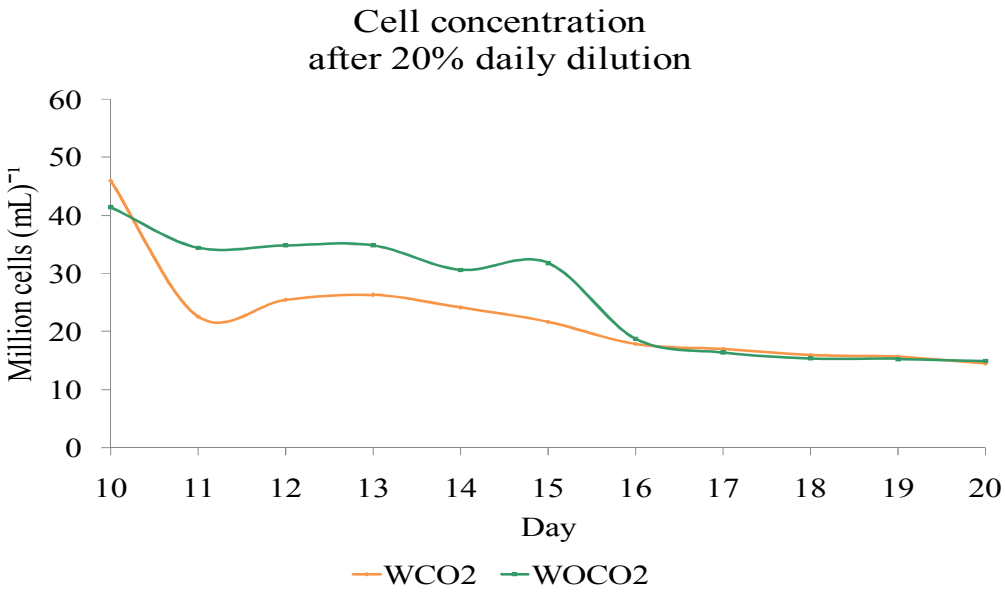


Figure 61. Cell concentration after 20% daily dilution.

Both treatments showed similar growth patterns observed through the FAU of water turbidity in both treatments; the turbidity values gradually descended according to the culture days due to the cell concentration (Figure 62) from 709 ± 164 on day 10 down to 216 ± 116 on day 20 in the treatment without CO_2. The cell concentration in the treatment with CO_2 fall from 722 ± 134 on the day 10 to 166 ± 47 until the day 20 was reached.

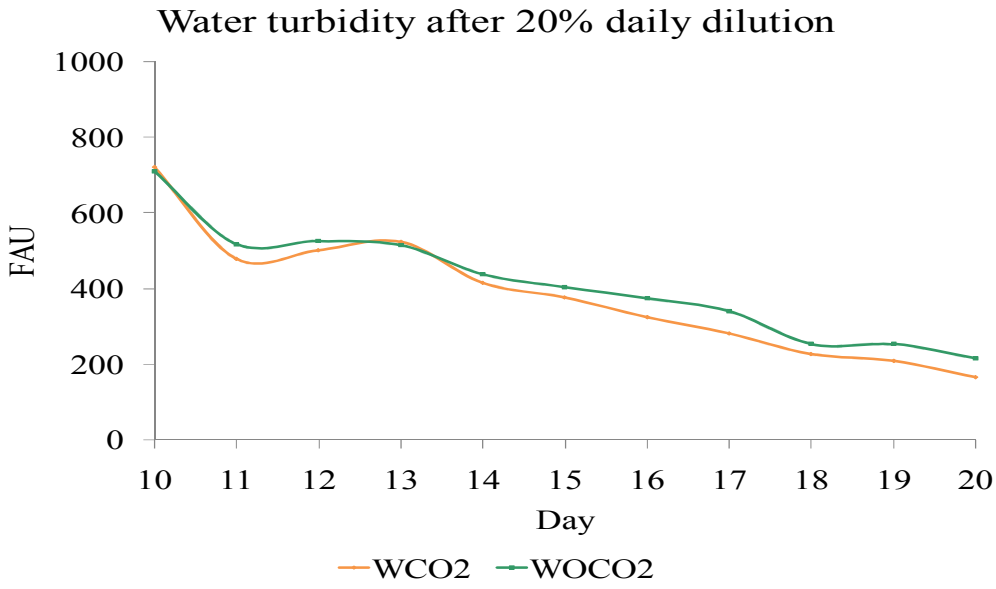


Figure 62. Water turbidity after 20% daily dilutions.

The correlation between cell concentration and water turbidity found on the treatment without CO_2 was R^2= 0.877 higher than the treatment with CO_2 with a correlated value of R^2=0.738 through the last ten days with a 20% daily dilution as presented in figure 63.

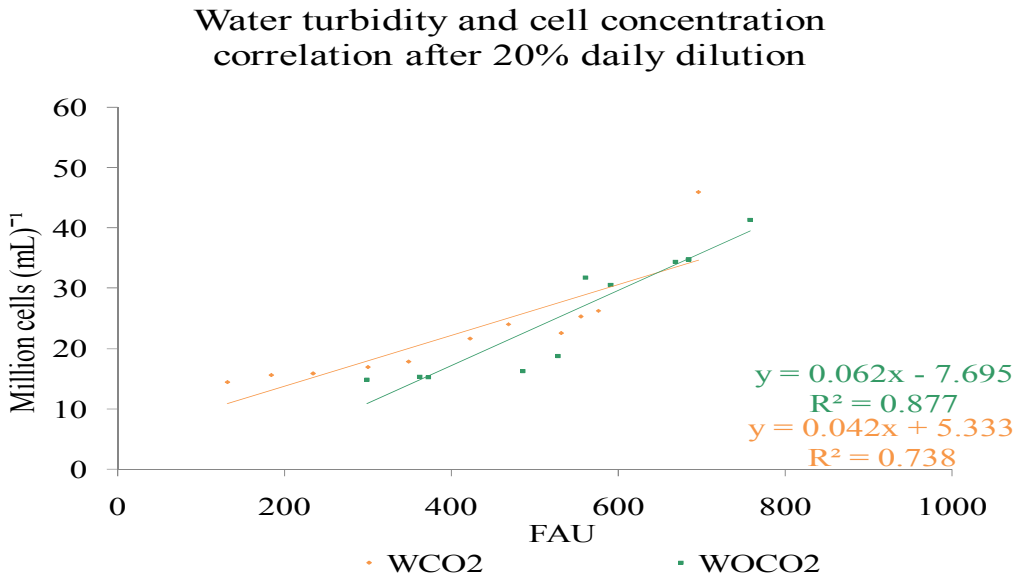


Figure 63. Correlation between water turbidity and cell concentration.

The pH values in both treatments were similar but the pH values were more stable around 7.55 ± 0.092 in the microalgae culture bags with CO_2 during the last ten days of culture, while in the ones without CO_2 pH showed values over 7.68 ± 0.168 but these values increased above 8 in the last 3 culture days. (Figure 64).

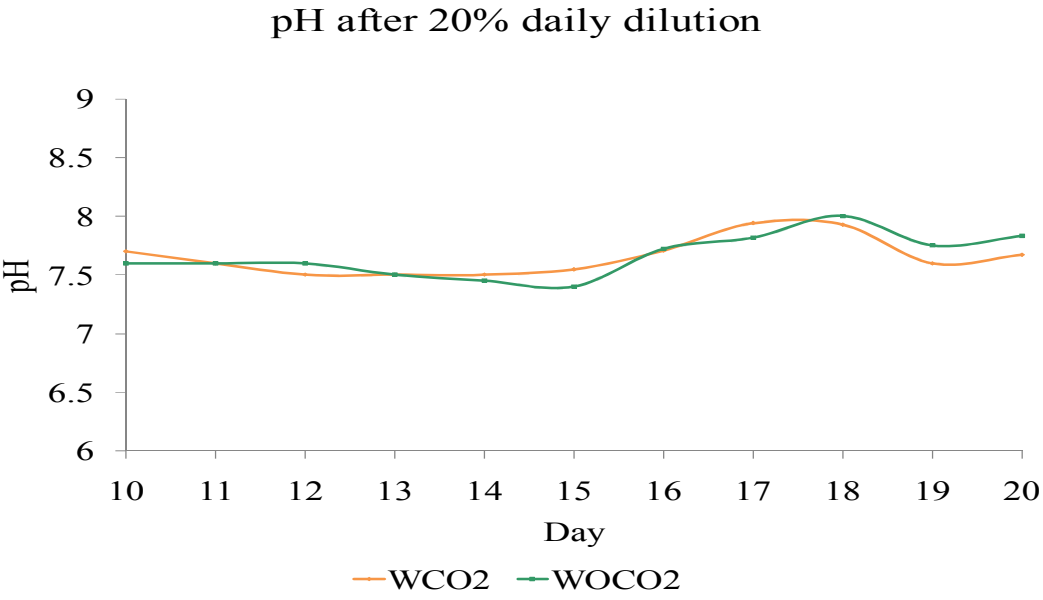


Figure 64. The pH values after 20% daily harvest.

4.2.4 Biochemistry results

The analytical proximal composition presented in figure 65 A, B and C showed that the moisture contain (%) in the microalgae paste samples was higher in the 3rd culture day in both treatments with 78.7 ± 2.7 in the treatment with CO_2 and 77.1 ± 3.78 in the treatment without CO_2, on the 10th culture day the moisture decreased in both treatments to 75.7 ± 2.66 in the treatment with CO_2, and 76.7 ± 0.78 in the treatment without CO_2. The moisture descended even more on the 20th culture day to 72.9 ± 3.8 in the treatment with CO_2, and 71.3 ± 4.19 in the treatment without CO_2. The lowest lipid concentration was observed in the third day as expected, followed by the 20th day sample. The highest lipid concentration was found on the 10th day of culture; although it was higher in the treatment with CO_2 the difference was not significant. The proteins had a similar behavior in both treatments with the highest content on the 3rd day of 58.2 ± 6.12 and 55.6 ± 12.56 decreasing to 47.7 ± 6.91 and 49.7 ± 9.51 on the 10th day ending on the lowest protein content which was 42 ± 10.83 and 41.8 ± 5.97 on the 20th culture day respectively in the treatments with and without CO_2 as shown on figure 65. The ash content showed the highest value on the 3rd day in the treatment with CO_2 with 6.64 ± 1.56 decreasing to 6.16 ± 2.68 on day 10 and the lowest value was observed in the 20th culture day with 2.31 ± 1.75 of ash content. By the other way in the treatment without CO_2 the highest value was observed in the 10th culture day with an ash content of 7.92 ± 4.31 followed by the 3rd culture day with 5.0 ± 2.92 and the lowest value was observed in the 20th culture day with an ash content of 2.2 ± 1.54 as shown on figure 65. The carbohydrate content values on the 3rd day were 14.2 ± 9.02 in the treatment with CO_2 and 20.37 ± 17.67 in the treatment without CO_2. On the 10th culture day the carbohydrate content climbed to 21.16 ± 9.72 in the treatment with CO_2, while the treatment without CO_2 decreased its carbohydrate content to 15.47 ± 14.14. At the 20th culture day the carbohydrate content increased in both treatments to 33.3 ± 11.33 and 34.56 ± 10.71 in the treatment with CO_2 and without CO_2 respectively.

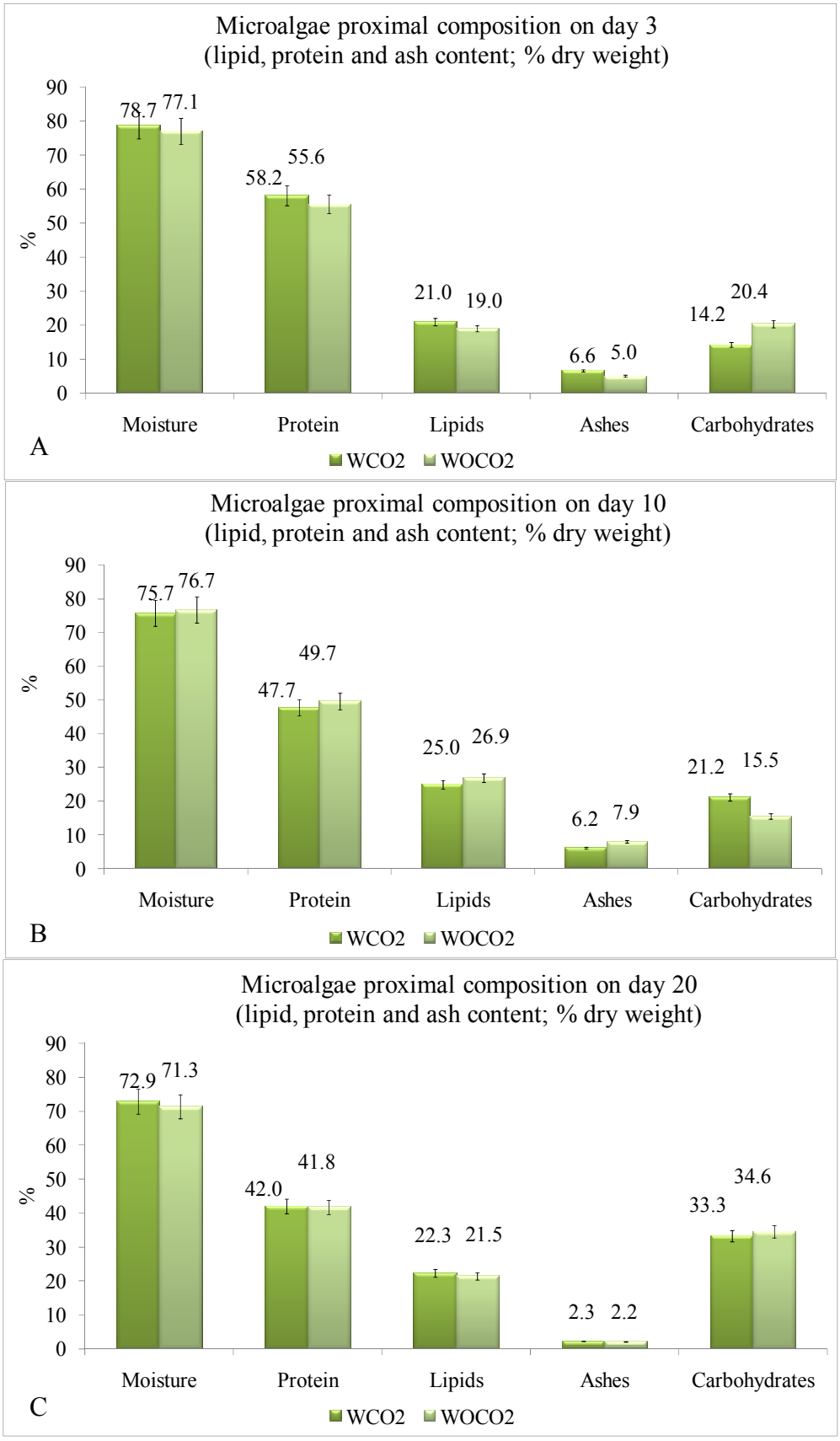


Figure 65. Microalgae (*N. gaditana*) proximal composition analysis on different culture days. A) on the 3rd day, B) on the 10th day and C) on the 20th culture day.

The microalgae fatty acids composition of *N. gaditana* is reported on table VI, where the saturated fatty acids of the treatment with CO_2 were highest at the 20th culture day with 7.649 ± 1.020 followed by the 3rd culture day with 7.031 ± 1.278 and the lowest value was on the 10th culture day and 6.297 ± 0.893. In contrast the treatment without CO_2 had the highest value of 8.151 ± 0.539 on the 10th culture day, followed by the 20th culture day with 7.782 ± 1.062 and the lowest value was on the 3rd culture day with 6.988 ± 1.354.

In the case of the mono-unsaturated fatty acids presented in table VI the highest value was reached at the 20th culture day with 8.592 ± 1.203 by the treatment with CO_2 which was lower on the 3rd and 10th culture day with 5.978 ± 2.244 and 5.635 ± 1.979 respectively. The other treatment without CO_2 had similar values between the three sample days with 7.394 ± 1.054 on the 10th culture day, 7.14 ± 2.959 on the 3rd culture day and 7.058 ± 1.528 on the 20th culture day. Oleic acid was higher on the 20th culture day with 1.201 ± 0.691 while in the 3rd and 10th culture day was 0.734 ± 0.197 and 0.743 ± 0.043 respectively.

The total n-3 HUFA`s are presented in figure 66 A. The treatment with $CO_2$ showed its higher content in the 3$^{rd}$ culture day with 5.796, decreasing to 3.931 in the 10$^{th}$ culture day and increasing again to 5.409 in the 20$^{th}$ culture day; in the treatment $WOCO_2$ the n-3 HUFA`s increased from 5.293 in the 3rd culture day to 5.618 in the 10th culture day reaching its higher content value of 5.976 in the 20th culture day.

The figure 66 B presents arachidonic acid content in the same sample days. The treatment without CO_2 showed its lowest ARA content on the 10th culture day (0.759) and its highest content in the 20th culture day (0.931); in contrast the treatment with CO_2 had the lowest ARA content the 3rd culture day (0.92), and the highest was on the 10th culture day (1.016). The figure 66 C showed eicosapentaenoic acid content. Almost all the total n-3 HUFA`s correspond to the EPA content of both treatments; in the treatment with CO_2 the highest EPA content was on the 3rd culture day with 5.529, while the lowest value was found on the 10th

culture day with 3.704; in contrast with the treatment without CO_2 which showed the lowest EPA content in the 3rd culture day with 4.964 and the highest value the 20th day with 5.785. The figure 66 D showed the docosahexaenoic acid content was contrary between the two treatments, in the treatment with CO_2 the lowest EPA content was on the 10th culture day with 0.189 and highest was found in the 20th culture day with 0.256; in contrast, the highest EPA content in the treatment without CO_2 was of 0.277 in the 3rd culture day and the lowest was 0.113 on the 20th culture day.

The relation between DHA and EPA content shown on table VI, showed the lowest content values in the treatment without CO_2 in the 20th culture day which were 0.023 ± 0.005, 0.034 ± 0.004 on the 10th culture day, and the highest value was 0.05 ± 0.014 on the 3rd culture day; while in the treatment with CO_2 the relation between this two fatty acids was bigger in the 10th culture day with 0.045 ± 0.012, followed by the 20th culture day with 0.041 ± 0.017 and 0.04 ± 0.009 on the 3rd culture day, this responds to the EPA content shown on figure 66 C.

The relation between the unsaturated fatty acids n-3 and n-6 presented in table VI showed the highest values on the treatment without CO_2 with 3.546 ± 0.032 on the 10th culture day, followed by the 20th culture day with 3.496 ± 0.101 and the lowest was 3.425 ± 0.317 on the 3rd culture day; meanwhile the treatment with CO_2 showed its mayor value on the 3rd culture day with 3.425 ± 0.317 followed by the 20th culture day with 3.151 ± 0.374, and he lowest value was on the 10th culture day with 2.93 ± 0.062.

Table VI. Fatty acid composition of both treatments on day 3, 10 and 20. Values are expressed in % of dry weight

	D3 WCO_2		D3 $WOCO_2$		D10 WCO_2		D10 $WOCO_2$		D20 WCO_2		D20 $WOCO_2$	
∑ Saturated[10]	7.031	±1.278	6.988	±1.354	6.297	±0.893	8.151	±0.539	7.649	±1.020	7.782	±1.062
∑ Mono- unsaturated[2]	5.978	±2.244	7.14	±2.959	5.635	±1.979	7.394	±1.054	8.592	±1.203	7.058	±1.528
∑ n-3[3]	5.888	±2.562	5.415	±2.691	4.01	±1.627	5.814	±0.946	5.542	±0.794	6.185	±0.420
∑ n-6[4]	1.686	±0.589	1.711	±0.709	1.363	±0.527	1.639	±0.252	1.699	±0.202	1.751	±0.093
∑ n-9[5]	0.786	±0.213	2.146	±0.514	2.285	±1.113	1.352	±0.073	1.329	±0.760	1.043	±0.364
∑ n-3 HUFA[6]	5.796	±2.551	5.293	±2.615	3.931	±1.597	5.618	±0.762	5.409	±0.687	5.976	±0.255
14:00	1.417	±0.146	1.202	±0.587	0.884	±0.288	1.567	±0.441	1.26	±0.819	1.648	±0.327
16:00	5.286	±1.069	5.459	±1.034	5.183	±1.297	6.202	±0.139	6.056	±0.062	5.793	±0.717
16:1 n-7	4.629	±1.959	4.571	±1.030	3.05	±1.313	5.648	±1.003	6.856	±1.070	5.493	±1.222
18:00	0.127	±0.020	0.178	±0.103	0.127	±0.075	0.192	±0.081	0.177	±0.046	0.131	±0.005
18:1 n-9	0.734	±0.197	1.058	±0.548	0.743	±0.043	1.283	±0.046	1.201	±0.691	0.992	±0.365
18:1 n-7	0.085	±0.019	0.09	±0.043	0.074	±0.046	0.113	±0.031	0.082	±0.057	0.11	±0.035
18:2 n-6	0.54	±0.171	0.58	±0.299	0.435	±0.230	0.355	±0.039	0.572	±0.423	0.461	±0.111
18:3 n-3	0.024	±0.007	0.037	±0.024	0.026	±0.022	0.013	±0.002	0.034	±0.033	0.019	±0.007
20:1 n-9	0.008	±0.002	0.032	±0.010	0.03	±0.014	0.021	±0.002	0.027	±0.009	0.014	±0.008
ARA	0.813	±0.300	0.92	±0.310	0.759	±0.194	1.016	±0.089	0.931	±0.437	0.946	±0.188
EPA	5.529	±1.512	4.964	±1.401	3.704	±1.410	5.39	±0.920	5.096	±0.379	5.785	±0.362
DHA	0.196	±0.035	0.277	±0.099	0.189	±0.082	0.163	±0.187	0.256	±0.277	0.113	±0.117
DPA (22:5n-6)	0.014	±0.004	0.018	±0.007	0.021	±0.007	0.015	±0.008	0.014	±0.002	0.012	±0.003
DHA/22:5 n-6	14.281	±2.754	17.058	±2.448	7.864	±1.819	8.73	±1.828	19.598	±2.414	8.738	±1.839
EPA/ARA	6.66	±0.663	5.209	±0.843	4.797	±0.631	5.285	±0.444	5.193	±1.191	6.13	±0.750
ARA/EPA	0.151	±0.016	0.195	±0.032	0.21	±0.028	0.19	±0.016	0.198	±0.045	0.165	±0.019
DHA/EPA	0.04	±0.009	0.05	±0.014	0.045	±0.012	0.034	±0.004	0.041	±0.017	0.023	±0.005
DHA/ARA	0.258	±0.092	0.272	±0.151	0.226	±0.182	0.169	±0.199	0.231	±0.189	0.143	±0.163
oleic/DHA	3.775	±0.804	5.976	±1.051	7.094	±1.597	22.781	±5.914	7.749	±1.671	22.44	±6.396
oleic/n-3HUFA	0.133	±0.026	0.206	±0.051	0.204	±0.072	0.231	±0.039	0.233	±0.031	0.168	±0.049
n-3/n-6	3.425	±0.317	3.092	±0.284	2.93	±0.062	3.546	±0.032	3.151	±0.374	3.496	±0.101

[10] (1) Includes 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 24:0; (2) Includes 14:1n-5, 14:1n-7, 16:1n-9, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-11, 22:1n-9 and 22:1n-7; (3) Includes 16:2n-3, 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3; (4) Includes 16:2n-6, 18:2n-6, 18:3n-6,18:4n-6, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-6, 22:3n-6, 22:4n-6 and 22:5n-6; (5) Includes 16:1n-9, 18:1n-9, 18:2n-9, 18:3n-9, 20:1n-9,20:2n-9, 20:3n-9 and 22:1n-9; (6) Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3.

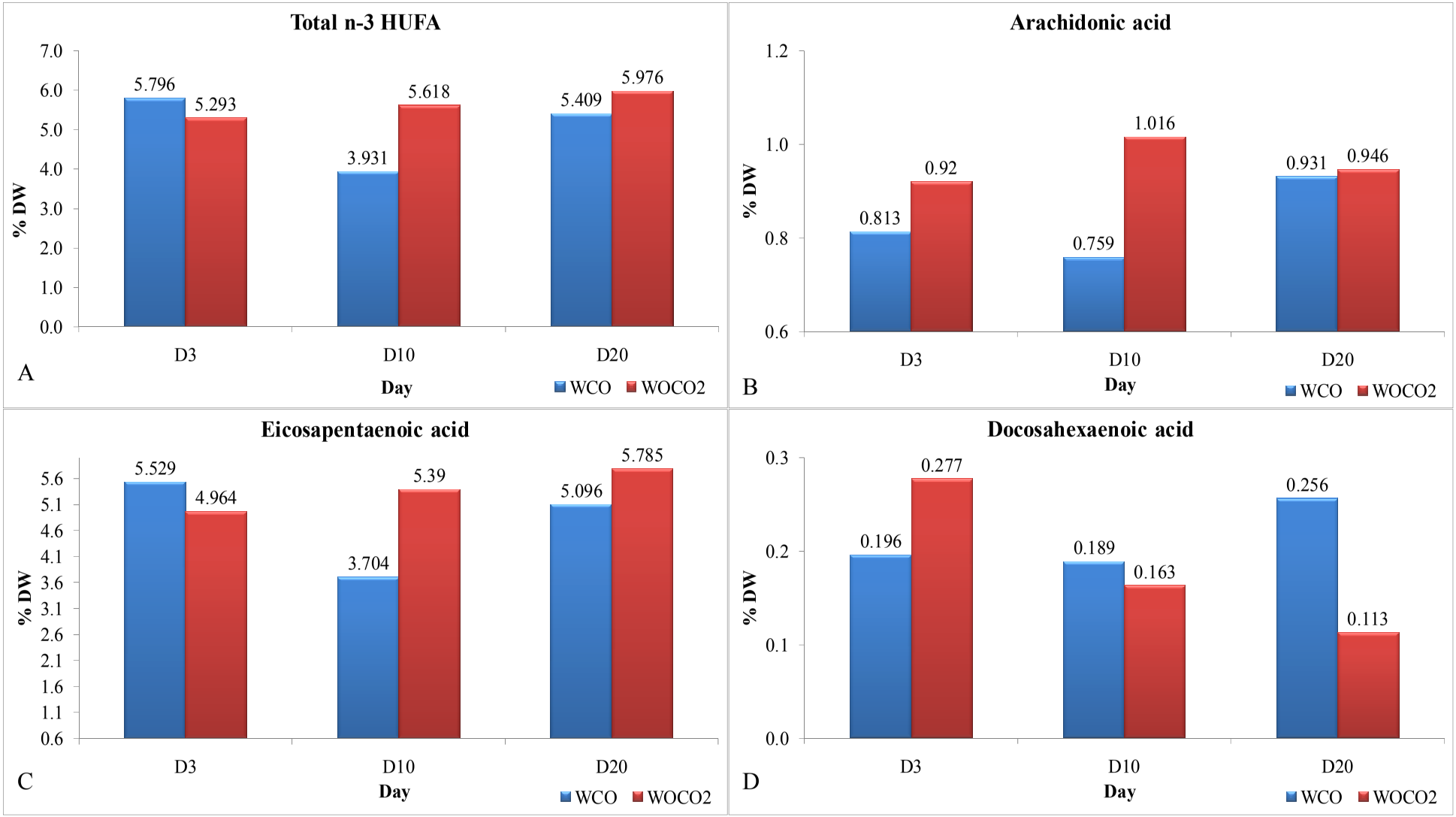


Figure 66. Fatty acids on three different sampling days: A) Total n-3 HUFA, B) Arachidonic acid content. C) Eicosapentaenoic acid content and D) Docosahexaenoic acid content in the 3rd, 10th and 20th culture days.

4.3 Vertical Photo-bioreactor (VPbr) preliminary trial.

4.3.1 Objectives

This experiment was made to improve *N. gaditana* cell density concentration by the achievement of outdoor vertical photo-bioreactors (VPbrs).

4.3.2 Materials and methods

One small (3.785 L) methacrylate vertical photo-bioreactor (VPbr S) settled up with recirculation water pump (Q=6.3 L $(min)^{-1}$) and air supply was installed next to two 50 L methacrylate vertical photo-bioreactors. One of them (VPbr I) had a recirculation water pump (Q=13.8 L·min^{-1}) and constant air supply, the other one (VPbr II) was equipped just with air supply and continuous CO_2 gas income as shown in figure 67. All three VPbrs where inoculated as mentioned on the Materials and Methods section and had twice nutrient income at the beginning of the culture. The systems began a daily harvest of 10% of the volume and replacing it with the same volume of enriched water from the 10^{th} to the 20^{th} culture day.

A B C

Figure 67. A) VPbrs settled outside the mesocosmo facilities at ICCM`s Hatchery. B) VPbr I equipped with a water pump and air supply. C) VPbr II equipped just with air supply and continuous CO_2 gas income.

4.3.3 Results

The cell concentration growth evaluation was interrupted due to pollution (*Cyanobacterium sp.*) in the three systems. The high temperatures recorded made easy the appearance of *Cyanobacterium sp.* in the VPbrs and the *N. gaditana* cells were displaced (Figure 68). Even under these circumstances the cell growth was estimated because *Cyanobacterium sp.* can be used as rotifer food too.

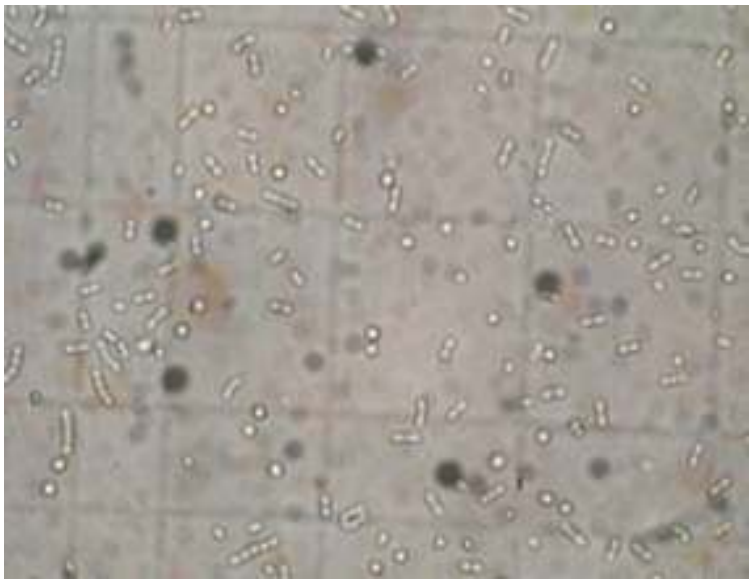


Figure 68. *N. gaditana* displacement by *Cyanobacterium sp*.

At the beginning of the test the *N. gaditana* cell concentration ($x10^6$ cells $(ml)^{-1}$) was 25 on the VPbr S, 18.06 on VPbr I and 17.25 on VPbr II. After the first two days of microalgae culture, the *N. gaditana* cells began growing as expected but at the third culture day the *Cyanobacterium sp.* pollution began the displacement of the *N. gaditana* cells growing exponentially up to high cell densities such as 818.81 in the VPbr II (with CO_2) on the 8^{th} culture day, this *N. gaditana* cell displacement also occurred in the other two VPbrs reaching high cell densities of 507.38 on VPbr I on day 14 and 492.94 on day 11 on the VPbr S as shown in figure 69.

At the same time, the displaced *N. gaditana* cells maintain a low concentration growth through all the experiment reaching the highest cell concentrations of 74.44 on the VPbr II

and 49.25 on the VPbr I, both on the 7th culture day and 44.81 on the VPbr S reached on day 10 (Figure 69).

Even though the microalgae culture didn`t worked as expected due to *Cyanobacterium sp.* pollution, the cell growth showed three phases (not very clear), the lag phase from day 0 to 2, followed by the exponential phase from day 3 up to the late exponential phase reached on the day 6 on the VPbr I, on day 8 on the VPbr II, and on the day 11 on the VPbr S.

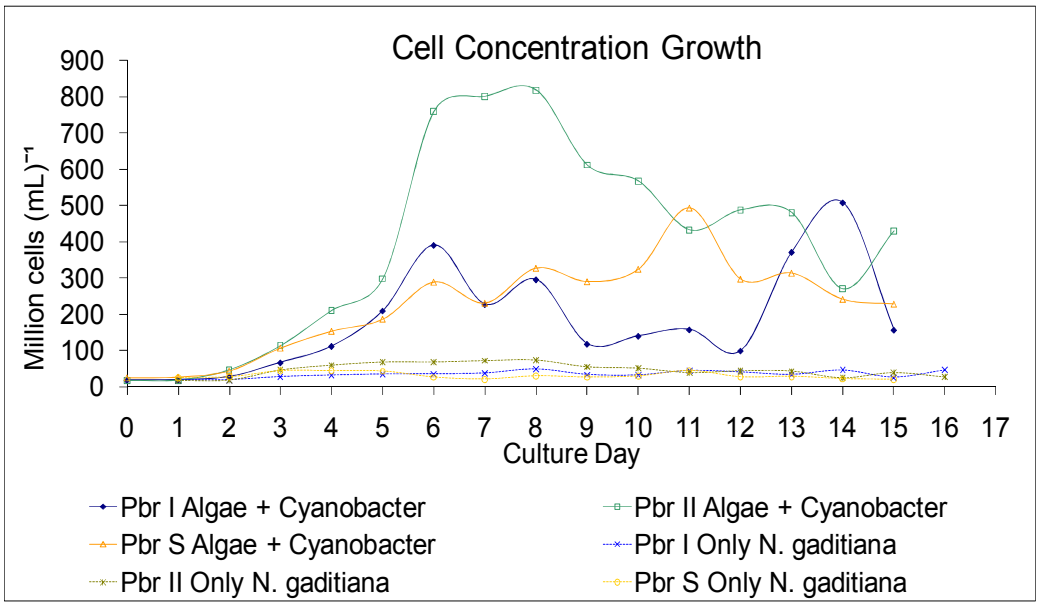


Figure 69. Outdoor VPbr`s cell concentration growth (*N. gaditana* plus *Cyanobacterium sp.*).

Similar growth patterns were observed with the FAU of water turbidity in the three VPbrs; the turbidity values ascend according to the culture days due to the cell concentration (*N. gaditana* and *Cyanobacterium sp.*) from 290 in VPbr I, 292 in VPbr II and 460 on VPbr S. at day cero up to the highest values of 1155 on VPbr I reached on day 8, 1995 on VPbr II on day 6 and 1252 on VPbr S reached on day 12 as shown in figure 70; Water turbidity (FAU) and cell concentration ($x10^6$ cells $(ml)^{-1}$) showed an acceptable correlation between their values in the VPbr II and Vpbr S along the experiment with a correlated value of $R^2= 0.757$ and $R^2= 0.701$ respectively while the VPbr I had no correlation at all with a $R^2= 0.062$ (Figure 71).

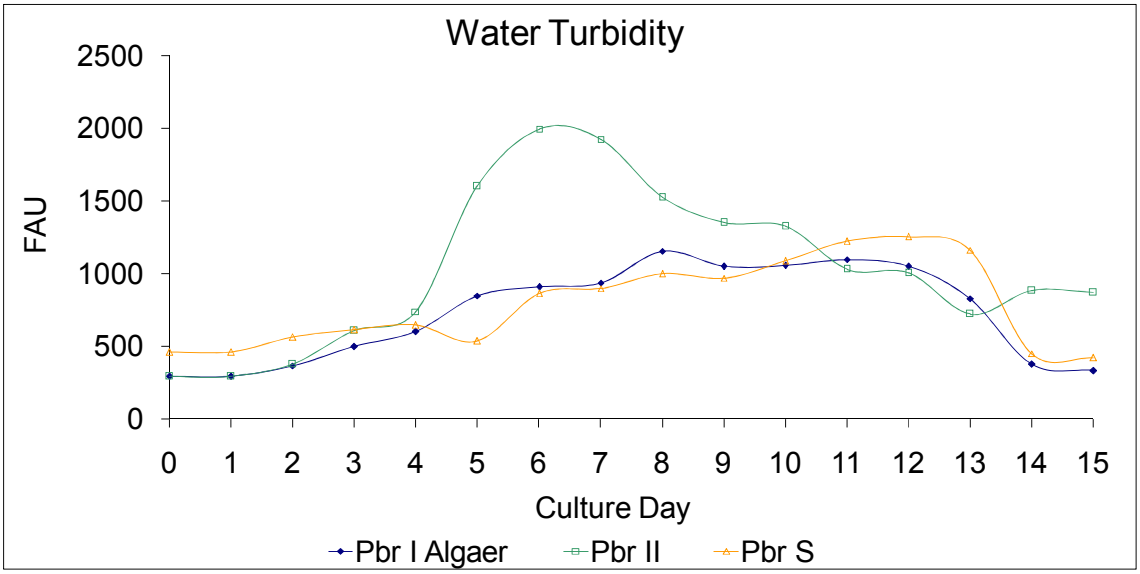


Figure 70 Water turbidity absorbance (FAU) of the three VPbrs through 15 culture days.

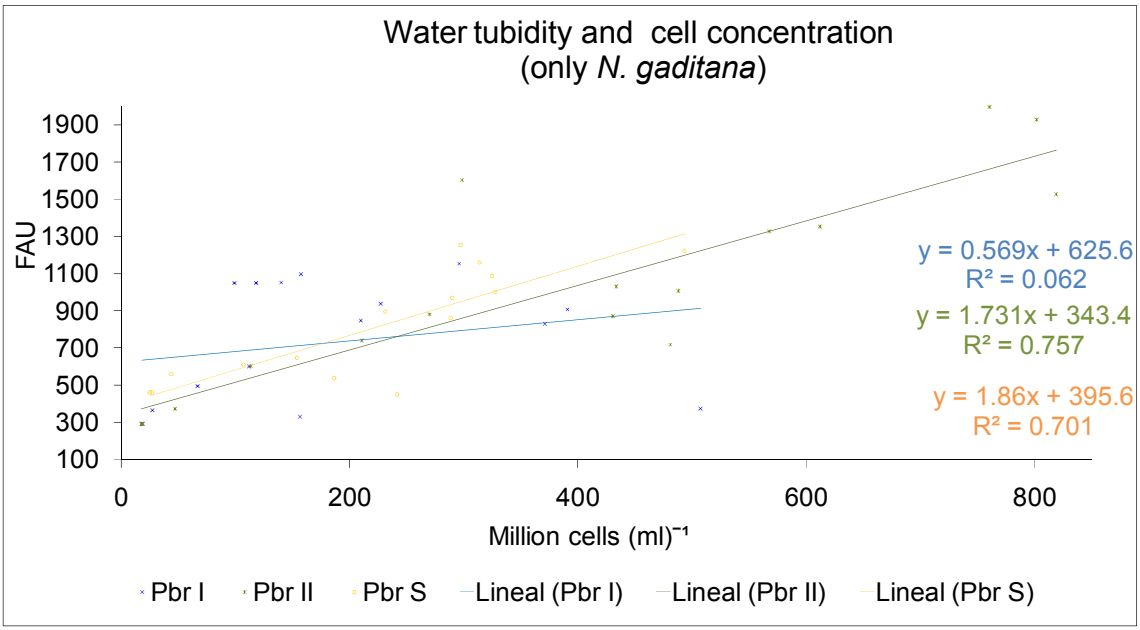


Figure 71. Correlation between cell concentrations and water turbidity

The pH values were more stable around 7.46 ± 0.38 in the VPbr II with CO_2 during the 15 days of culture, while the pH values of VPbr II and VPbr S (the ones without CO_2) increased from 7.4 on day 0 to 10.29 on day 6 and from 7.1 on day 0 to 10.1 on day 6 respectively., with some oscillations on day 11 and 12 of culture, (Figure 72).

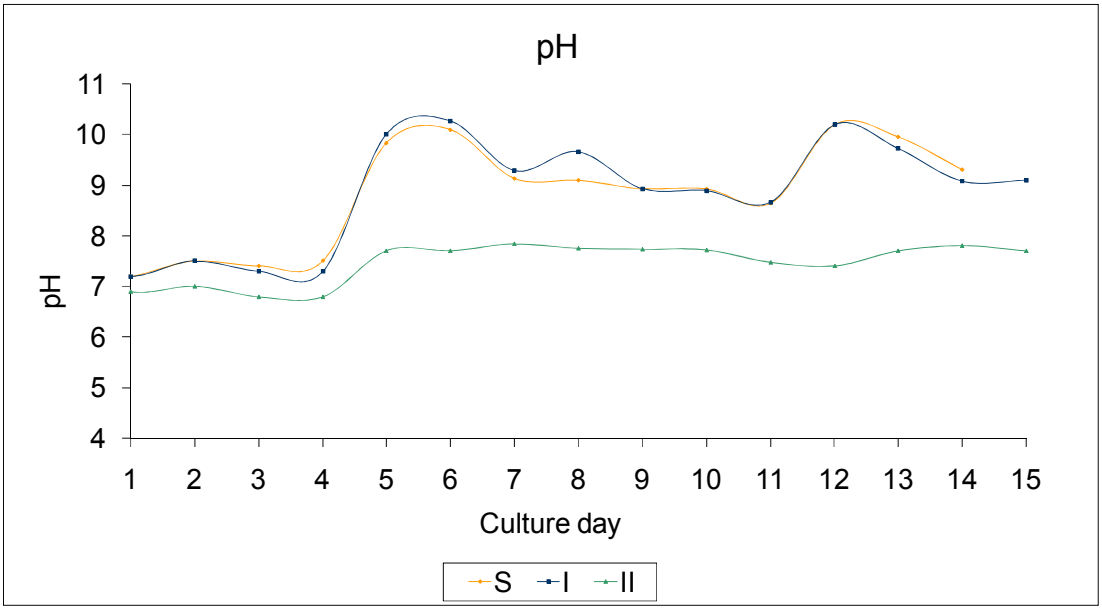


Figure 72. pH values of the VPbrs during the experiment three.

As the dissolved oxygen was daily measured at the same time (09:00 hrs), every measure reported oxygen concentrations higher than 6 mg $(L)^{-1}$. The VPbr I and II had a dissolved oxygen concentration increase on the 6th, 7th and 8th culture day up to 8.80 $mg(L)^{-1}$ and 9.20 $mg(L)^{-1}$ respectively, this concentration decreased from day 9 to 15 down to 7.30 $mg(L)^{-1}$ on VPbr I and II, and 7.7 $mg(L)^{-1}$ on VPbr S (Figure 73). Every day the oxygen was above 6 $mg(L)^{-1}$.

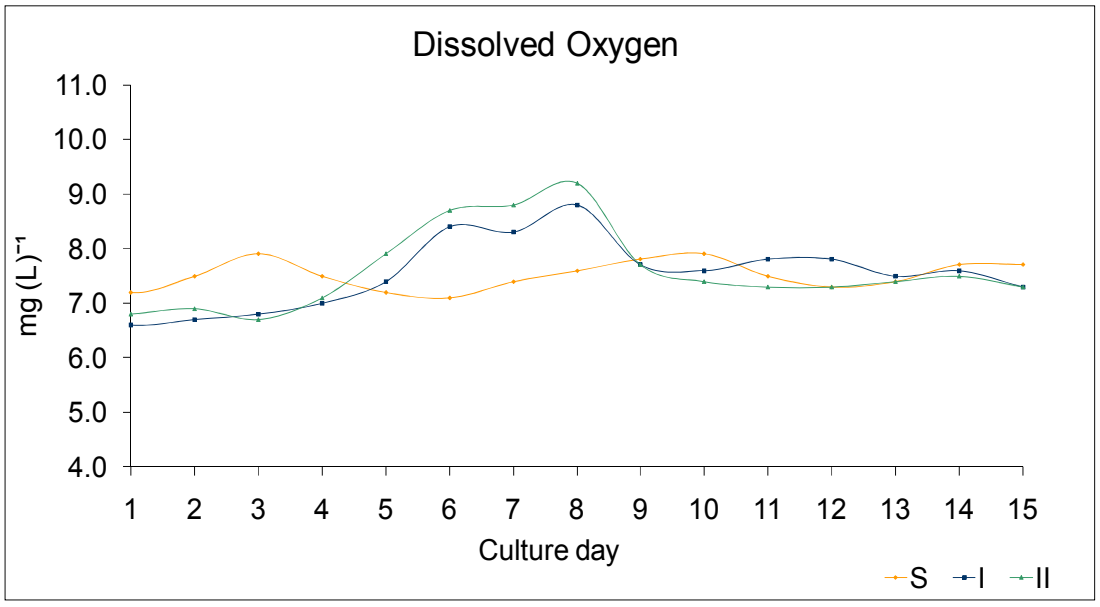


Figure 73. Dissolved oxygen on the three outside Vpbrs through 15 days of culture.

As oxygen measurements, temperature and light intensity[11] were measured daily at the same time; the temperature variations were a response of the water culture to the constant weather changes, where a sunny day highly influences the temperature (ºC) and light intensity (Lux) measured on the VPbrs.

At the beginning of the experiment a cloudy day provoke low measures of light intensity (4600 Lux) in the VPbrs, while the temperatures measured at the same time were 33ºC on VPbr I, 34 on VPbr II and 34.9 on the VPbr S.

The light intensity (Lux) average on the three VPbrs was 60306 ± 26755 with the highest value of 98700 reached on day 8 (Figure 74) which was a very sunny day. The highest temperature (ºC) average was 36.44 ± 3.32 in the VPbr S, while the highest temperature recorded of 40ºC was reached at days 5 and 8. The average temperature (ºC) of the VPbr I and II was 32.97 ± 2.68 and 34.59 ± 3.35 respectively; the highest temperature was 37 on VPbr I and 39 on VPbr II reached at the same days as shown in figure 74 A and B.

[11] The same values of light intensity correspond to the three VPbrs because they received the same illumination as they stand one next to another in the same place and position.

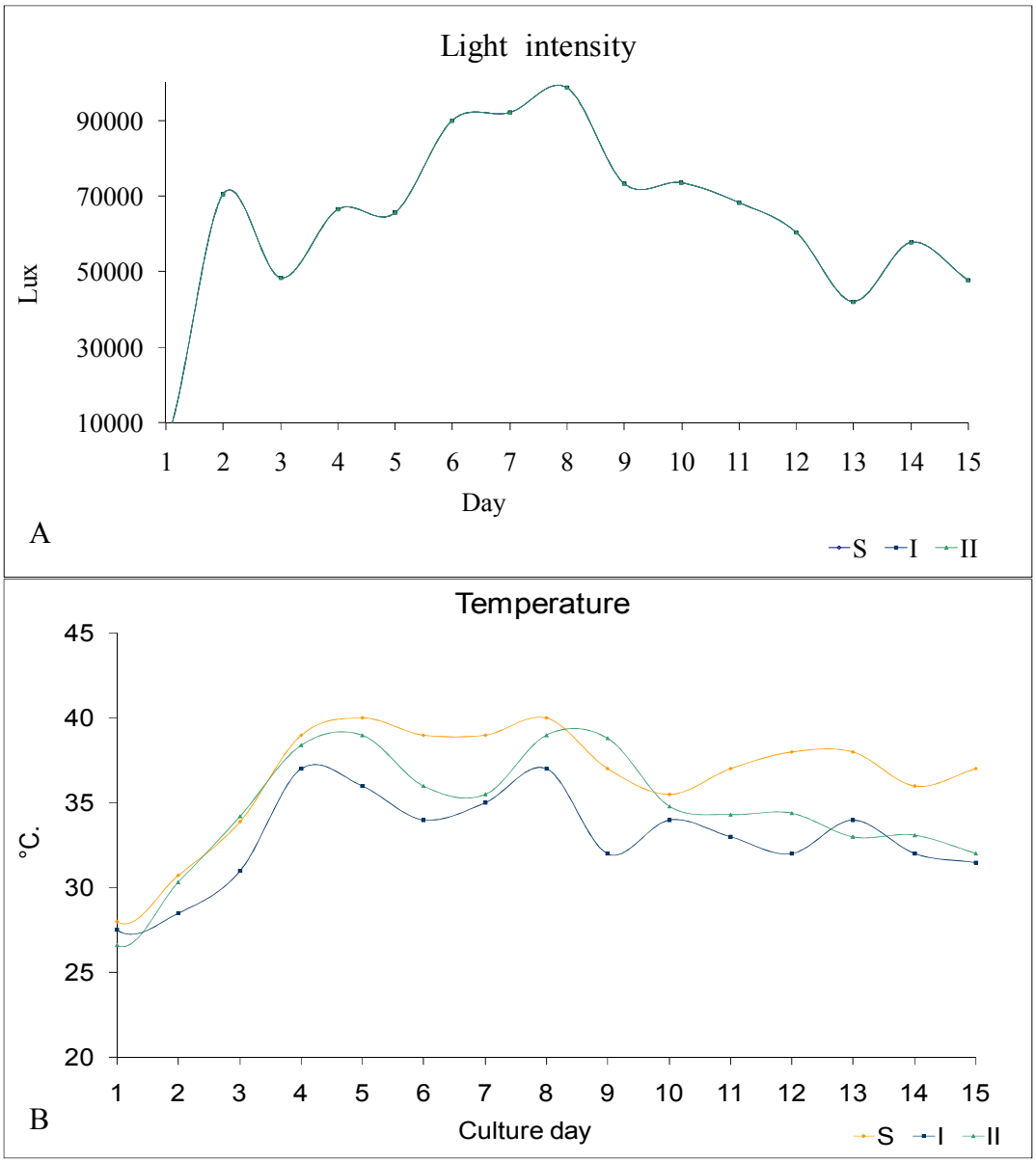


Figure 74. A) Light intensity in the three VPbrs. B) Culture temperature during the 15 day culture experiment.

At the beginning of the experiment the water used for the microalgae culture was enriched (twice dose) for the VPbrs; the initial nitrate concentration (mg $(L)^{-1}$) was 60 on VPbr S, 61.3 on VPbr I and 62.4 on VPbr II.

During the first 10 days of culture the nitrate concentration values decreased as the nutrients were consumed down to 25 on VPbr S, 50 on VPbr I and 30 on VPbr II. On the ninth day previous the 10% daily dilution began.

After dilutions started, dissolved nitrate values recovered and consumed every day due to the replacement of enriched water. The VPbr I showed a more constant pattern of nutrient add-consume through the last five days of the experiment with a nitrate concentration (mg $(L)^{-1}$) mean of 53.57 ± 5.79, while the VPbr S and VPbr II were more oscillating in the dissolved nitrate values with a mean concentration of 34.48 ± 13.14 and 44.44 ± 12.7 respectively but kept the same model (Figure 75).

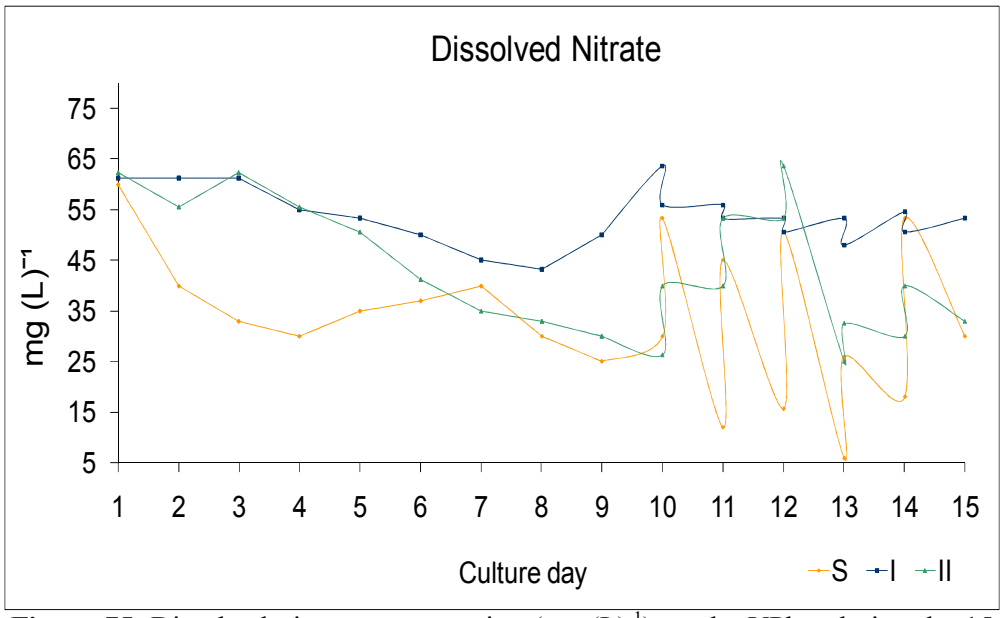


Figure 75. Dissolved nitrate concentration (mg $(L)^{-1}$) on the VPbrs during the 15 day culture experiment.

4.4 Experiment three

4.4.1 Objectives

This experiment was done to improve *N. gaditana* cell growth through vertical Photo-bioreactors (VPbrs) against traditional culture bag for 30 days and 10% daily harvest.

4.4.2 Materials and methods

In accordance with the previous experiment the cell concentration improvement took place in the outside vertical Photo-bioreactors. Due to *Cyanobacterium sp.*pollution, the outdoors VPbrs were tested again and compared with a traditional 50L culture bag inside the microalgae room to improve the cell concentration growth with the following changes:

The 50 L bag was traditionally cultured, started with twice nutrients (2XN) added to the culture water at the beginning of the experiment, there was no CO_2 gas income, just the regular bubble diffuser aeration.

The VPbr I was started with twice nutrients (2XN) added to the water at the beginning of the experiment. There was no re-circulation water pump used this time, just the regular bubble aeration.

The VPbr II was started with four times nutrients (4XN) and there was no CO_2 gas income, just the regular bubble aeration.

N. gaditana was cultured through 30 days on two 50 L volume Vertical Photo-bioreactors and one 50 L bag. The two VPbrs and the bag were inoculated at the same time, starting from an initial cell concentration ($x10^6$ cells $(ml)^{-1}$) of 21.2 ± 3.3 on day zero.

4.4.3 Results

The microalgae growth showed three phases in the three systems at the first 10 days of culture without manipulation. The lag phase occurred on day 0 and day 1 followed by the exponential growth phase from day 3 to day 9 reaching the late exponential phase at the day

10 with cell concentrations of 160 ± 36.51 in the VPbr I, 158.1 ± 37.58 in the VPbr II and 65 ± 8.2 in the microalgae bag reached on day 11 (Figure 76).

After 10 days without manipulating the systems, a semi-continuous culture began in the three systems with 10% volume dilution, every day 5 L were harvested and replaced with enriched water at the same proportions that they were started; VPbr I and bag with twice nutrient dose while the VPbr II with four times the nutrient income. The culture bag began to show diatoms stocked inside its surface (Figure 77).

Past 20 days of microalgae culture the cell concentration kept growing on both VPbrs and reached 161.88 ± 25 in the VPbr I and 213.8 ± 12.91 in the VPbr II, while the cell concentration of the bag decreased down to 30 ± 9.3.

By this point the microalgae bag was getting full of diatom pollution attached to the walls inside it which contributed to obstruct the light intensity inside the bag and the cell concentration decrease (Figure 78 A, B and C). On the other side the VPbrs still had clean their surfaces which allow the light intensity kept flowing inside and reached the microalgae cells as shown in figure 79 A, B and C.

The highest cell concentration on the VPbr I was 271.88 ± 9.59 reached on the 27^{th} culture day, the VPbr II reached its higher cell concentration of 317.5 ± 24.49 on the 28^{th} day, while the bag kept decreasing from day 10 ending with a cell concentration of 12.44 ± 4.03 on the 30^{th} culture day as shown in figure 77.

In this experiment no significant differences were observed between the cell concentration growths of the two VPbrs, but there was a significant difference ($P>0.05$) between the cell concentration growth of the culture bag and both VPbrs at the first ten days of culture. After the 10% daily dilutions there was a significant difference ($P>0.05$) between each VPbr and also between both VPbrs and the culture bag.

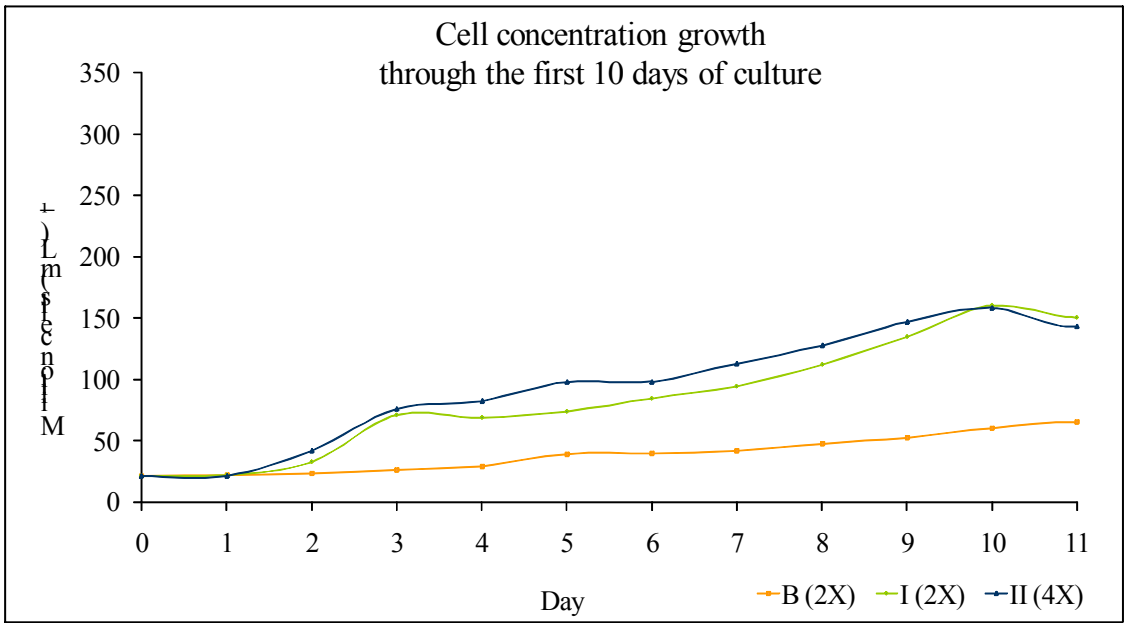


Figure 76. Cell concentration growth through 10 days of culture.

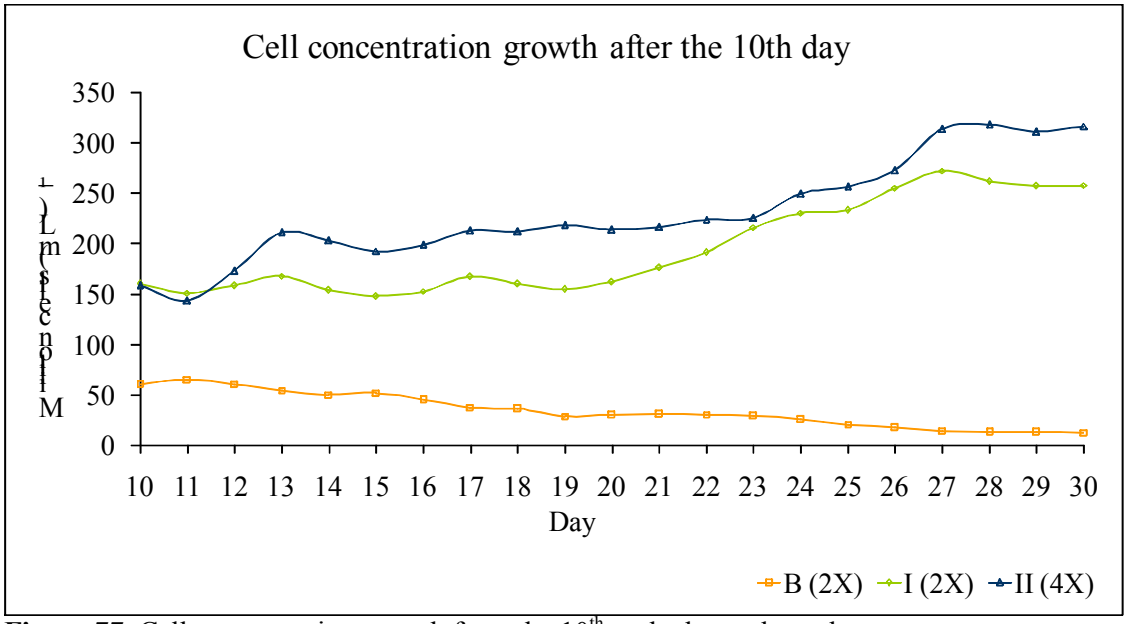


Figure 77. Cell concentration growth from the 10th to the last culture day.

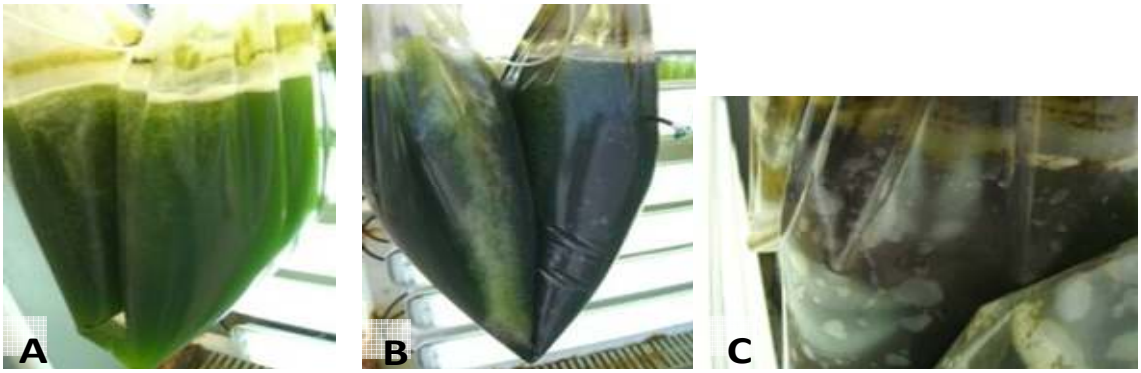


Figure 78. Evolution of diatom pollution attached inside the microalgae culture bag avoiding the light intensity to penetrate inside the bag. A) Day 10, B) Day 20 and C) day 30.

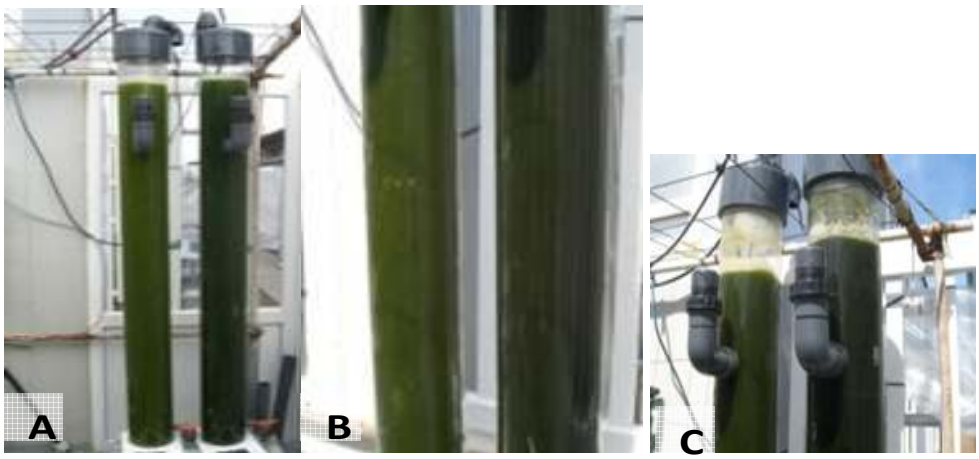


Figure 79. Evolution of the organic matter attached inside the methacrylate VPbrs. A) At day 10 the VPbrs. B) At day 20. C) At day 30.

The microalgae biomass daily production (g $(L)^{-1}$ average from day 10 to day 30 was 0.36 ± 0.18 in the culture bag, with significant difference (P>0.05) against both VPbrs. The VPbrs biomass production was 3.71 ± 0.86 in VPbr I and 4.39 ± 0.97 in VPbr II on the same days with significant differences (P>0.05) between them as shown on figure 80.

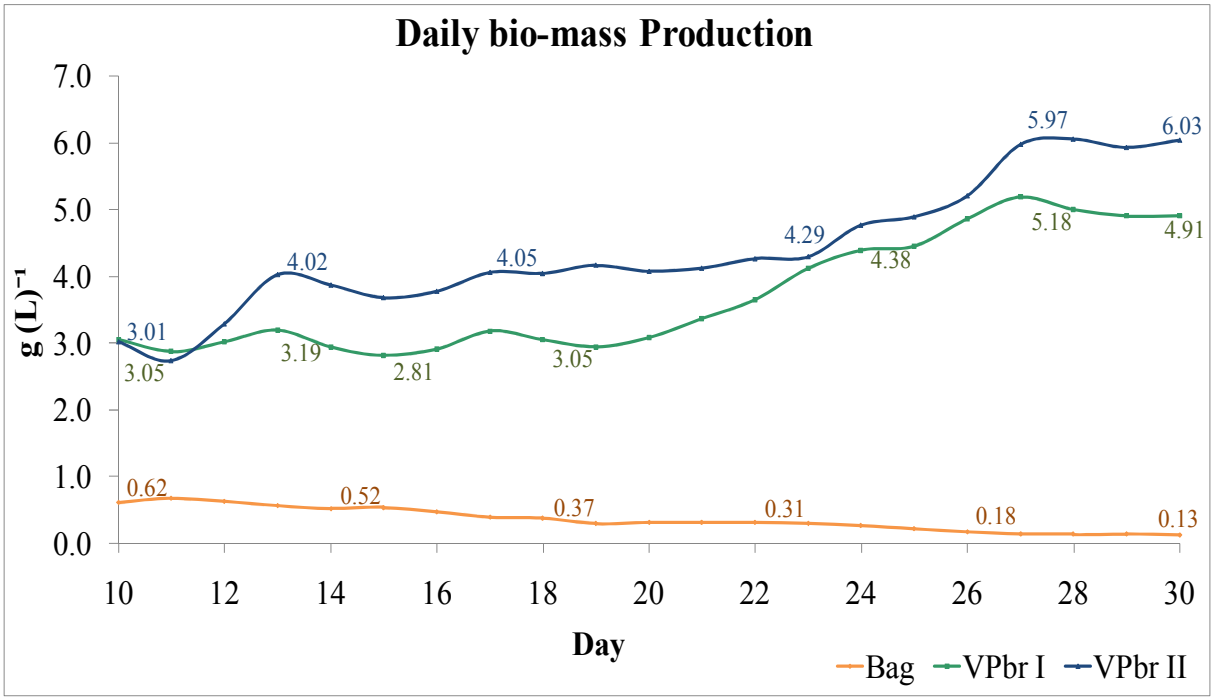


Figure 80. Microalgae production after 10% daily harvest.

Two similar growth patterns were observed with the FAU of water turbidity in both VPbrs and culture bag; the turbidity values ascend according to the culture days due to the cell concentration (Figure 81) from 210 in both VPbrs and culture bag on day 0 up to 2140 on VPbr I, 2240 on VPbr II and 1100 on the culture bag at the 10th culture day.

After the 10% daily dilutions began on day 10, FAU kept the same pattern than the cell concentration, increasing to 3680 on the VPbr I, 3550 on the VPbr II and decreasing to 410 on the culture bag.

The highest absorbance reached by the VPbr I was 4680 on day 27, 5590 by the VPbr II on the 28th day and the culture bag decreased to 205 on the 30th as shown in figure 81.

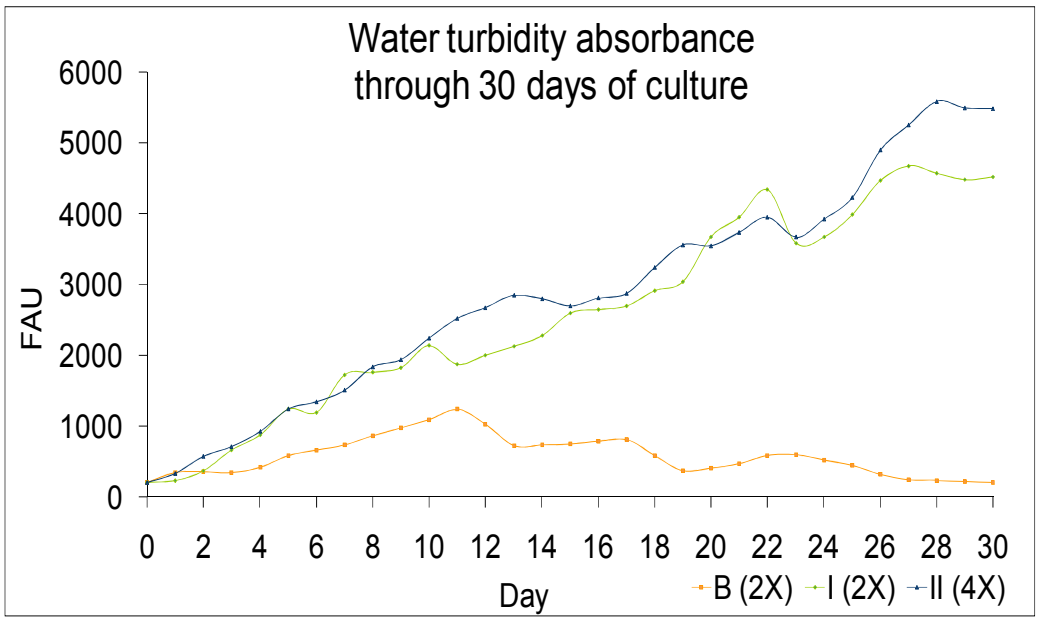


Figure 81. Water turbidity on the VPbrs and on the culture bag through 30 days.

Water turbidity and cell concentration showed a high correlation between their values along the experiment in all systems. The VPbr I had correlated values of R^2= 0.907, the VPbr II had a correlated value of R^2=0.970 while the culture bag showed a correlated value of R^2=0.897. This correlation was reached through 30 days of culture (Figure 82).

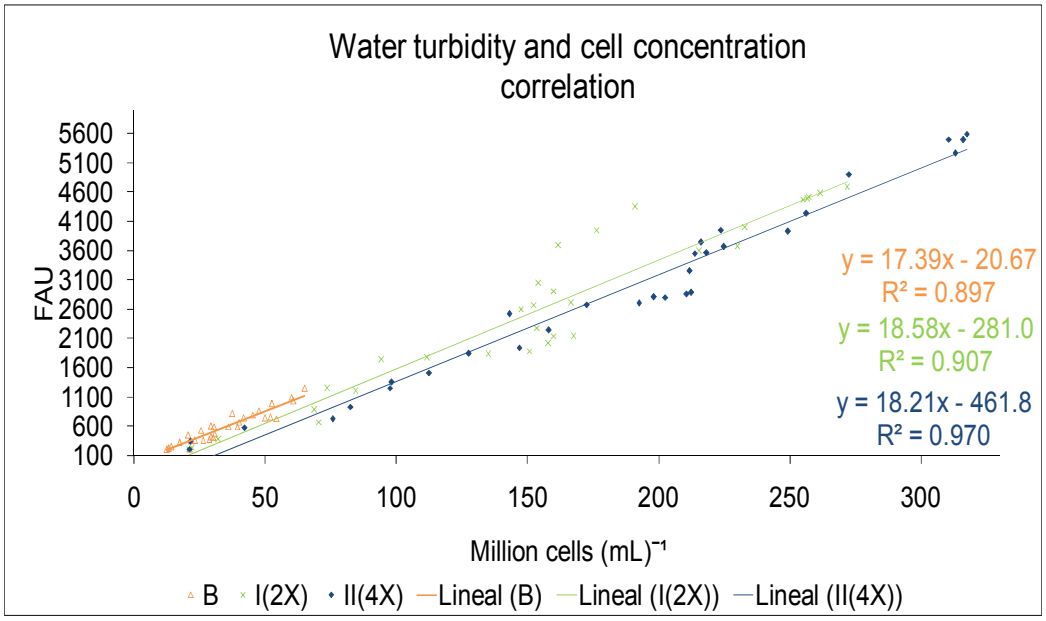


Figure 82. Water turbidity and cell concentration correlation.

The initial pH in the microalgae culture began on 8.4 at day 0 on each system, the culture bag quickly increased its pH up to 10.4 on day 6 decreasing again to 9.5 at day 10 where the daily dilutions began, the pH remain stable near 10 until day 22 increasing up to day 30 up to 10.9 as shown in figure 83.

The VPbrs (I and II) showed a similar behavior between each other, pH increased on day 1 up to 8.8 in both VPbrs, decreasing on day 2 to 8.7 and 8.5 respectively, oscillating near 9 till the daily dilutions began on the 10th culture day with pH values of 9.3 and 9.2; reaching its higher pH value on day 15 with 9.9 on VPbr I and 10 on Vpbr II, decreasing again down to 8.7 and 9.1 on day 17, gradually increasing up day 30 with pH values of 9.8 on VPbr I and 10 on VPbr II.

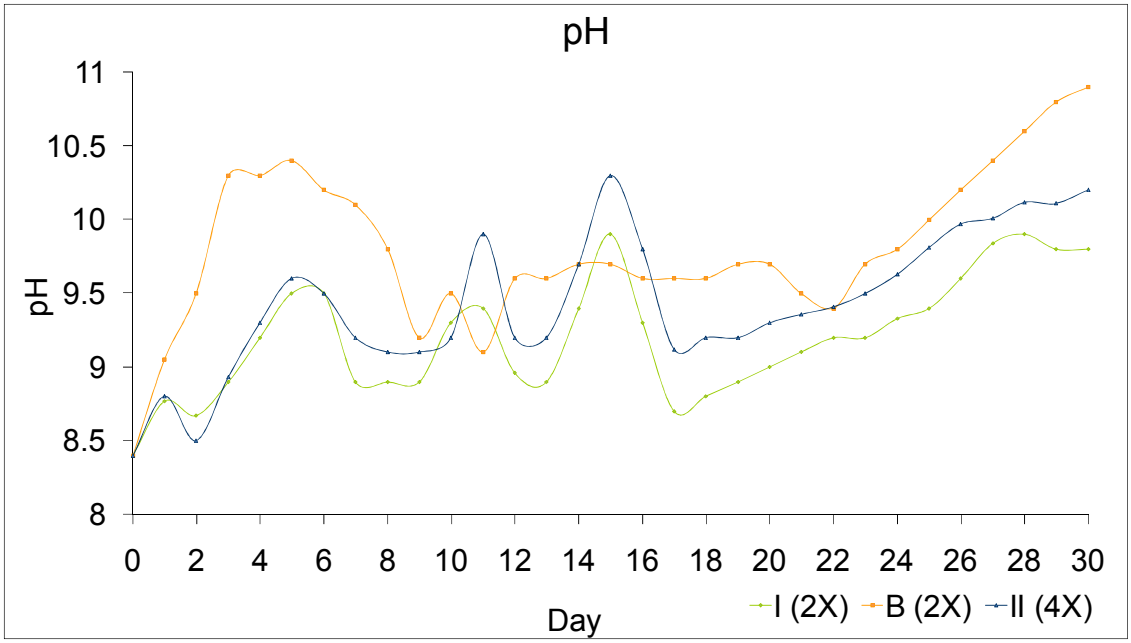


Figure 83. The pH evolution during 30 days of experiment.

At the beginning of the experiment the water used for the microalgae culture was enriched (twice dose) for the bag and VPbr I while the Vpbr II was enriched with the quadruple (4X) the regular nutrient dose; the initial nitrate concentration (mg $(L)^{-1}$) was 95 on the culture bag and 120 in both VPbrs. During the first 7 days of culture the nitrate concentration values decreased as the nutrients were consumed to 5 on the culture bag and VPbr I, and 74.1 on VPbr II. As the nitrate concentration was very low nutrients were added again in the same proportions that at the beginning of the experiment, increasing again the dissolved nitrate values up to 65 on the culture bag 50 on VPbr I, and 130 on VPbr II on the 8^{th} day decreasing again until the 10^{th} day was reached and the dilutions began**.**

After the 10% daily dilutions started, figure 84 showed that the dissolved nitrate values gradually increased until the 14^{th} day with 40 on the culture bag, 70 on VPbr I and 108.5 on VPbr II; gradually decreasing until day 22 when the dissolved nitrate concentrations were 40 on the culture bag, 30 on VPbr I and 40 on VPbr II. One more time nutrients were added again in the same proportions that at the beginning of the experiment, increasing again the dissolved nitrate values up to 55 on the culture bag, 65 on VPbr I and 100.4 on VPbr II on the

23rd day gradually decreasing until the last day of the experiment was reached with ending nitrate values of 35 on the culture bag, 65 on VPbr I and VPbr II.

All three systems showed a similar pattern of nutrient add-consume through the experiment with a nitrate concentration ($mg(L)^{-1}$) mean of 44.61 ± 19.78 on the culture bag, 49.96 ± 23.52 on VPbr I and 89.13 ± 25.21 on VPbr II as shown in figure 84.

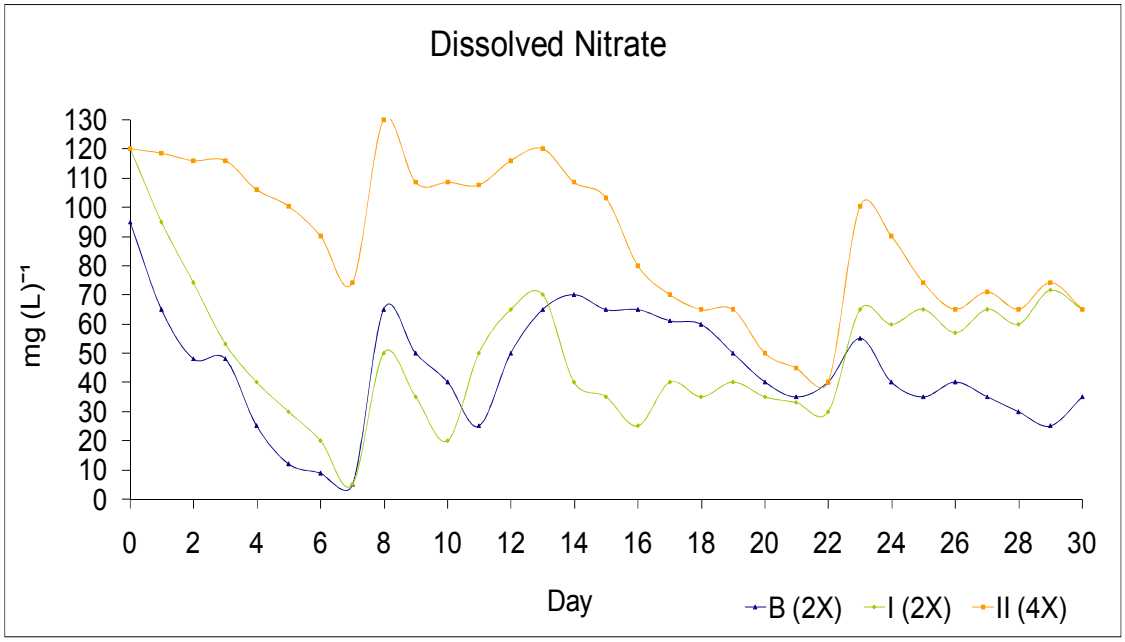


Figure 84. Dissolved nitrate concentration during 30 days of experiment.

Temperature and light intensity[12] were measured daily at the same time; the temperature variations were a response of the water culture to the constant weather changes, where a sunny day highly influences the temperature (ºC) and light intensity (Lux) measured on the VPbrs; while the bag inside the microalgae room received very low light intensity from the sun (Figure 85).

At the beginning of the experiment the light intensity was 83400 lux in the VPbrs, while the culture bag began the experiment receiving 3100 lux of light intensity the same day (Figure 85). The light intensity average on both VPbrs was 83194 ± 15704 with the highest

[12] The same values of light intensity correspond to Both VPbrs because they received the same illumination as they stand one next to another in the same place and position.

value of 99800 reached on day 27 which was a very sunny day, while the light intensity average on the culture bag was 3610 ± 930.7 with a highest value of 5600 reached on day 25 as shown in figure 85.

Temperatures measured at the same time were 22 ºC on VPbr I and II, and 23ºC on the culture bag (Figure 86).

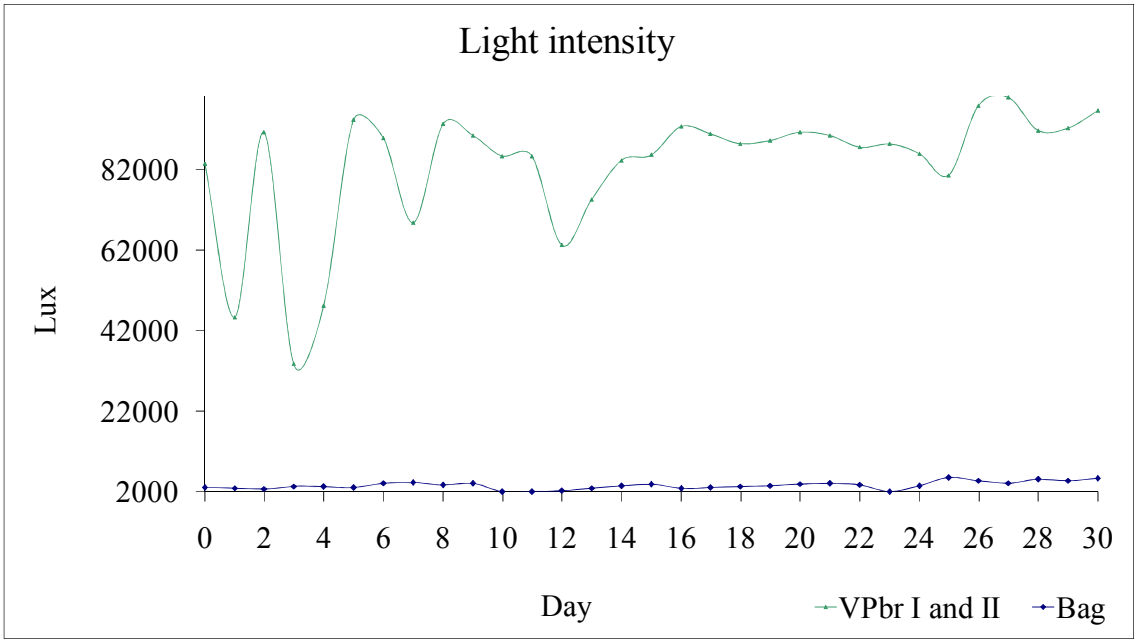


Figure85. Light intensity in both VPbrs and in the culture bag.

The highest temperature (ºC) average was 24 ± 2.447 in the VPbr I, followed by the VPbr II with an average temperature of 23.87 ± 2.55; the highest temperature recorded of 29ºC was reached at days 26, 27 and 30 in both VPbrs. The average temperature (ºC) of the culture bag was 23.65 ± 1.78 with a maximum of 27 reached at days 25, 26, 29 and 30 (Figure 86).

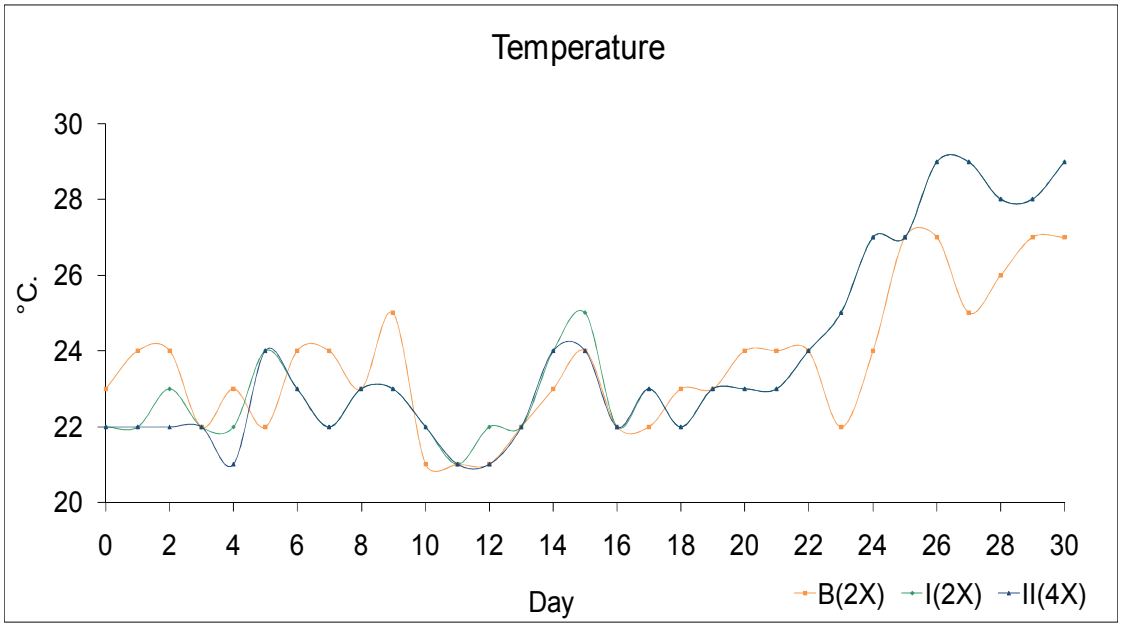


Figure 86. Temperature of the VPbrs and culture bag through 30 days of experiment.

4.4.4 Biochemistry results

The moisture content (%) decreased from 83.5 to 82.1 in the VPbr I, from 85.11 to 80.31 on VPbr II from day 10 to day 30, while the moisture increased in the culture bag from 76.4 to 76.9 in the same days (Figure 87 A and B).

In the three samples there was an increase of the proteins (%DW) from day 10 to day 30 from 32.3 to 56.3 in the culture bag, from 44.3 to 48.y in the VPbr I and from 48.62 to 61.45 in the VPbr II (Figure 87 A and B).

The same pattern than the moisture content was showed inversely in the lipid content with a lipid decrease in the culture bag from 13.3 to 9, while the VPbrs increased their lipid content from 10.7 to 12.1 in VPbr I and from 5.98 to 18.33 in VPbr II in the same days (Figure 87 A and B).

The ash content increased from day 10 to day 30 in the culture bag from 4.5 o 4.8, while the VPbrs decreased on the same days from 7.5 on VPbrI and from 15.78 to 9.14 on VPbr II (Figure 87 A and B).

The three systems had higher carbohydrate content (% DW) on day 10 than day 30; the culture bag decreased from 49.9 to 30, the VPbr I from 37.5 to 32.6 and the VPbr II from 29.62 to 11.07 (Figure 87 A and B).

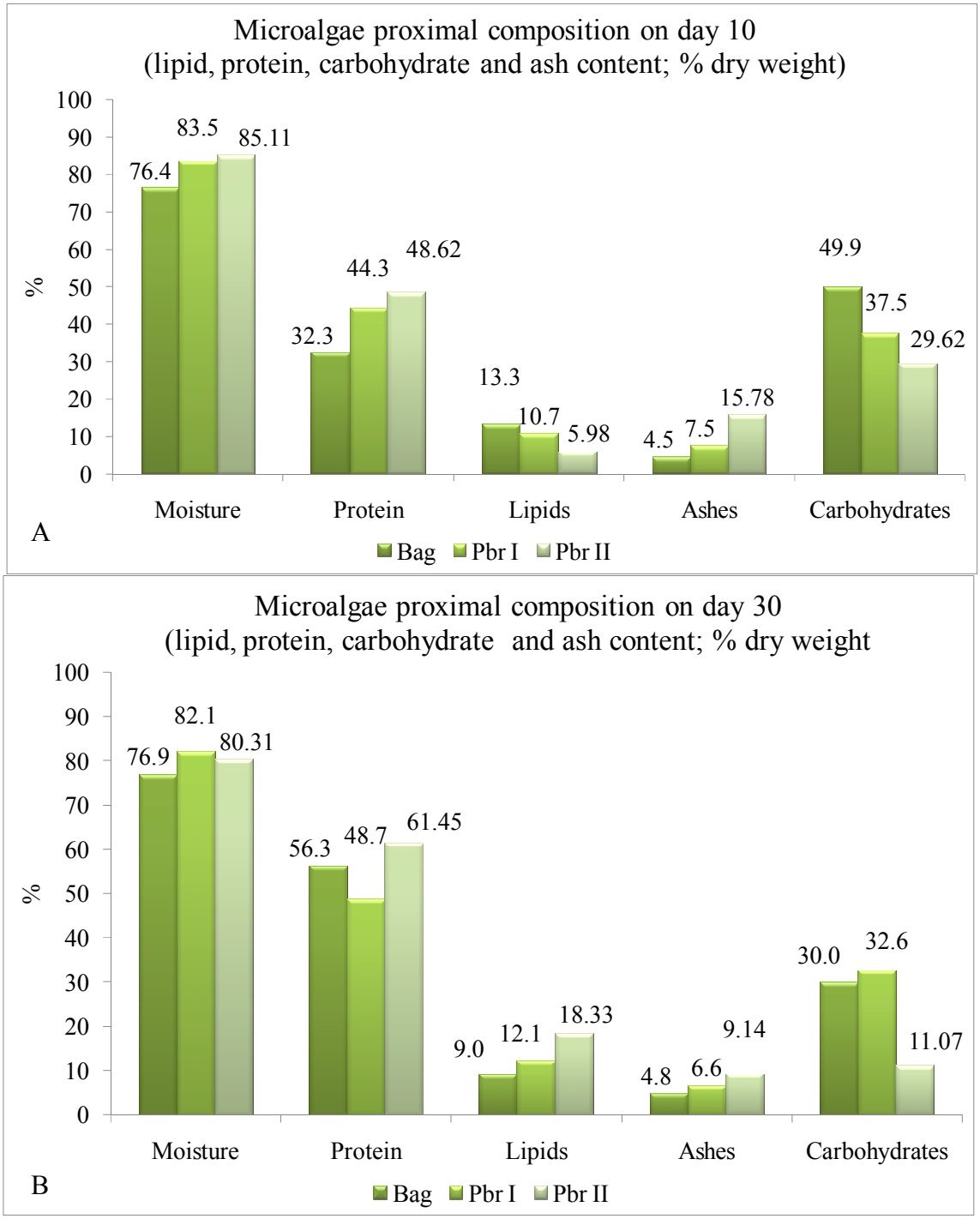


Figure 87. Microalgae (*N. gaditana*) proximal composition analysis on different culture days. A) on the 10rd day, B) on the 30th culture day.

The microalgae fatty acids composition of *N. gaditana* is reported on table VII, values are presented in % of dry weight.

Saturated fatty acids were higher in the 30th culture day in the three systems; the highest value was obtained by the VPbr II with 4.836, followed by the Vpbr I with 3.655 and the culture bag had the lowest saturated acids content of 2.177 as shown in table VII. The same pattern occurred with the mono-unsaturated fatty acids with the highest content in the 30th culture day, in the same proportions than the saturated fatty acids, this time the highest value was obtained by the VPbr II with 4.620, followed by VPbr I with 3.681 and the culture bag at last with 1.918. Oleic acid had its highest value in the VPbr I with 0.553, followed by the VPbr II with 0.475 and the culture bag with 0.373 in the 30th culture day; the lowest values obtained at the 10th culture day kept the same proportions with 0.447 in VPbr I, 0.223 in VPbr II and there was no oleic acid detected in the culture bag at the 10th culture day.

The total n-3 HUFA content on the 10th culture day shown in figure 88 A were higher with 3.262 in VPbr I followed by the culture bag with 2.775 and the VPbr II at last with 2.513; on the 30th culture day (Figure 88 B) the VPbr II increased to 6.928 followed by the culture bag with 4.027 and the VPbr I with 3.26. Almost the total n-3 HUFA content correspond to the EPA content which was higher in the VPbr I with 3.102, followed by VPbr II with 2.482 and the culture bag at last with 1.196 (Figure 88A). At the 30th culture day the VPbr II had the highest EPA content of 6.843 followed by the culture bag with 3.971 and the VPbr I at last with 3.231 (Figure 88 B). At the 10th culture day ARA was higher in the VPbr I with 0.447, followed by the VPbr I with 0.254 and there was no ARA found in the culture bag sample (Figure 88 A). By the 30th culture day the ARA highest vale was found in the VPbr II with 0.557 followed by the culture bag with 0.405 and the VPbr I at last with 0.281(Figure 88 B). The minor n-3 HUFA found in every sample was DHA with the highest content of 0.026 on VPbr I followed by VPbr II and there was no DHA in the culture bag at the 10th culture day

(Figure 88 A); this low proportion remain until day 30 when the highest content still was in the VPbr I with 0.011, this time followed by the culture bag with 0.018 and the VPbr II at last with 0.014 as shown in figure 88 B.

Once again the EPA was the main n-3 acid in the samples with almost all total HUFAs composed by EPA, which lead to a very small DHA/EPA relation in all samples; this relation was null in the culture bag on day 10, because there was no DHA found on it but the VPbr I relation on the 10^{th} culture day was 0.008 against 0.003 on the VPbr II; in contrast this same samples changed their DHA/EPA proportion through the 30^{th} culture day, when the highest value was obtained by the culture bag with 0.004, followed by the VPbr I with 0.003 and at last the VPbr II with 0.002 as shown in table VII. The relation between n-3 and n-6 acids was always above 1.0 due to the high content of EPA in all samples which leads to a higher n-3 proportion, however this relations changed from day 10 to day 30. On the 10^{th} culture day the highest relation was obtained by the VPbr II with 5.664, 3.681 in VPbr I and 1.766 in the culture bag, while the same treatments on the 30^{th} culture day showed a higher value in the culture bag with 5.750, followed by VPbr II with 4.878 and 4.21 in VPbr I as table VII shows.

Table VII. Fatty acids composition of the three tested systems on culture day 10 and 30

	10th Day			30th Day		
	Bag	**VPbr I**	**VPbr II**	**Bag**	**VPbr I**	**VPbr II**
∑ Saturated[13]	0.898	3.046	1.307	2.177	3.655	4.836
∑ Mono-unsaturated[2]	1.689	3.348	1.626	1.918	3.681	4.620
∑ n-3[3]	5.051	3.343	2.538	4.064	3.715	7.234
∑ n-6[4]	2.860	0.908	0.448	0.707	0.882	1.483
∑ n-9[5]	1.546	0.468	0.242	0.412	0.568	0.493
∑ n-3HUFA[6]	2.775	3.262	2.513	4.027	3.260	6.928
14:00	0.171	0.507	0.251	0.431	0.634	1.195
16:00	0.263	2.380	0.991	1.632	2.854	3.381
16:1 n-7	0.288	2.650	1.309	1.390	2.891	3.509
18:00	0.463	0.074	0.024	0.039	0.068	0.044
18:1 n-9	0.000	0.447	0.223	0.373	0.553	0.475
18:1 n-7	0.499	0.075	0.027	0.033	0.062	0.063
18:2 n-6	0.572	0.236	0.091	0.150	0.515	0.659
18:3 n-3	0.691	0.060	0.017	0.026	0.385	0.266
20:1 n-9	0.000	0.010	0.003	0.006	0.002	0.010
ARA	0.000	0.447	0.254	0.405	0.281	0.557
EPA	1.196	3.102	2.482	3.971	3.231	6.843
DHA	0.000	0.026	0.007	0.018	0.011	0.014
DPA (22:5n-6)	0.000	0.033	0.008	0.000	0.000	0.005
DHA/22:5 n-6	0.000	0.780	0.972	0.000	0.000	2.752
EPA/ARA	0.000	6.938	9.754	9.816	11.499	12.294
ARA/EPA	0.000	0.144	0.103	0.102	0.087	0.081
DHA/EPA	0.000	0.008	0.003	0.004	0.003	0.002
DHA/ARA	0.000	0.058	0.029	0.044	0.039	0.025
oleic/DHA	0.000	17.336	29.809	21.143	49.930	33.821
oleic/n-3HUFA	0.000	0.137	0.089	0.093	0.170	0.069
n-3/n-6	1.766	3.681	5.664	5.750	4.210	4.878

[13] (1) Includes 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 24:0; (2) Includes 14:1n-5, 14:1n-7, 16:1n-9, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-11, 22:1n-9 and 22:1n-7; (3) Includes 16:2n-3, 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3; (4) Includes 16:2n-6, 18:2n-6, 18:3n-6,18:4n-6, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-6, 22:3n-6, 22:4n-6 and 22:5n-6; (5) Includes 16:1n-9, 18:1n-9, 18:2n-9, 18:3n-9, 20:1n-9,20:2n-9, 20:3n-9 and 22:1n-9; (6) Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3.

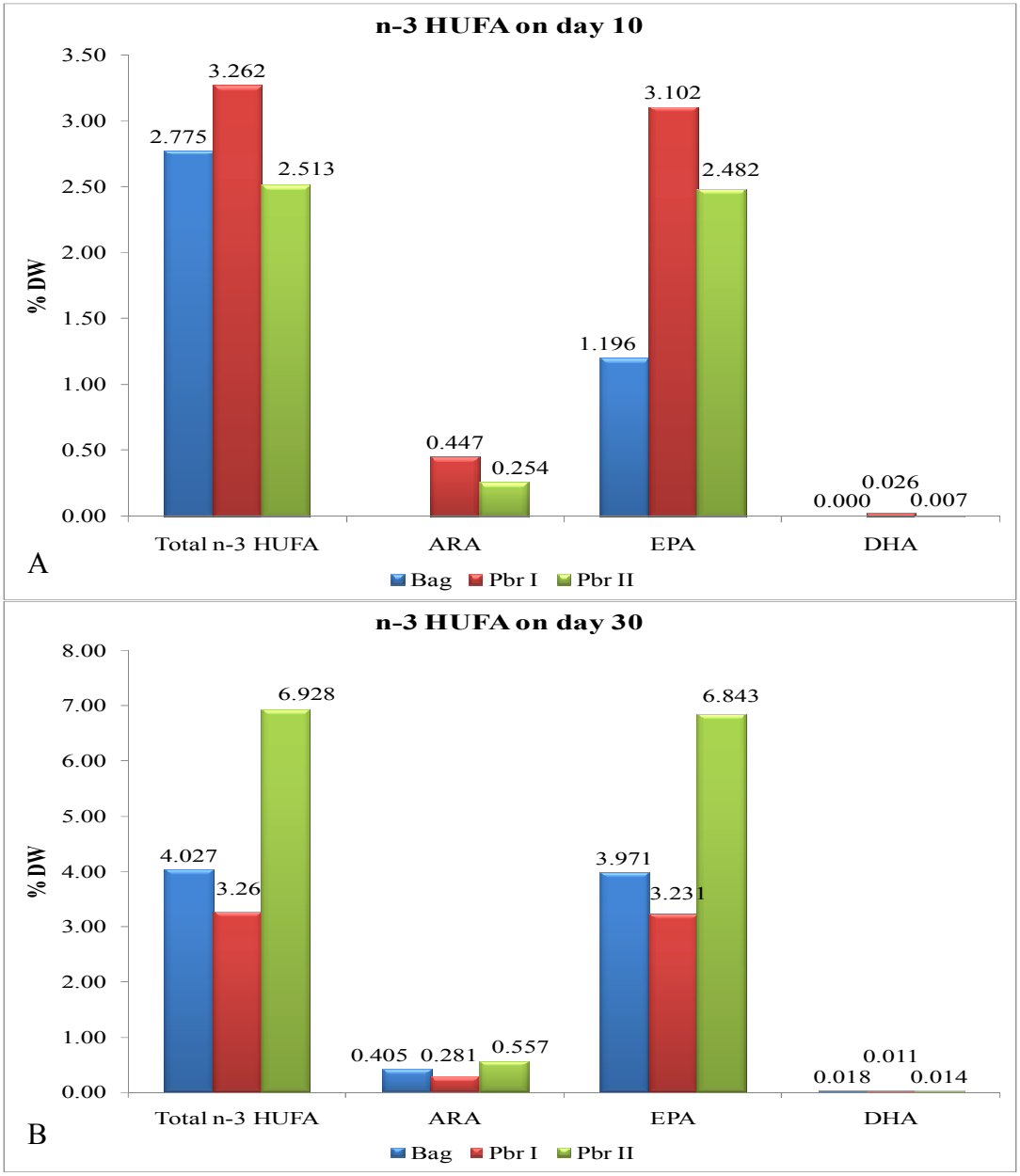


Figure 88. n-3 HUFA`s evolution from day 10 (A) to 30 (B).

4.4.5 Bacteriology results

The visible direct count (VDC) showed a bacterial increased (CFU (100 ml)$^{-1}$ from day 10 to day 30, from 1.38 (x 10^5) to 4.4 (x 10^6) in the culture bag, from 2.06 (x 10^5) to 9 (x 10^5) in VPbr I and from 3.48 (x 10^5) to 4.12 (x 10^6) on VPbr II in figure 89.

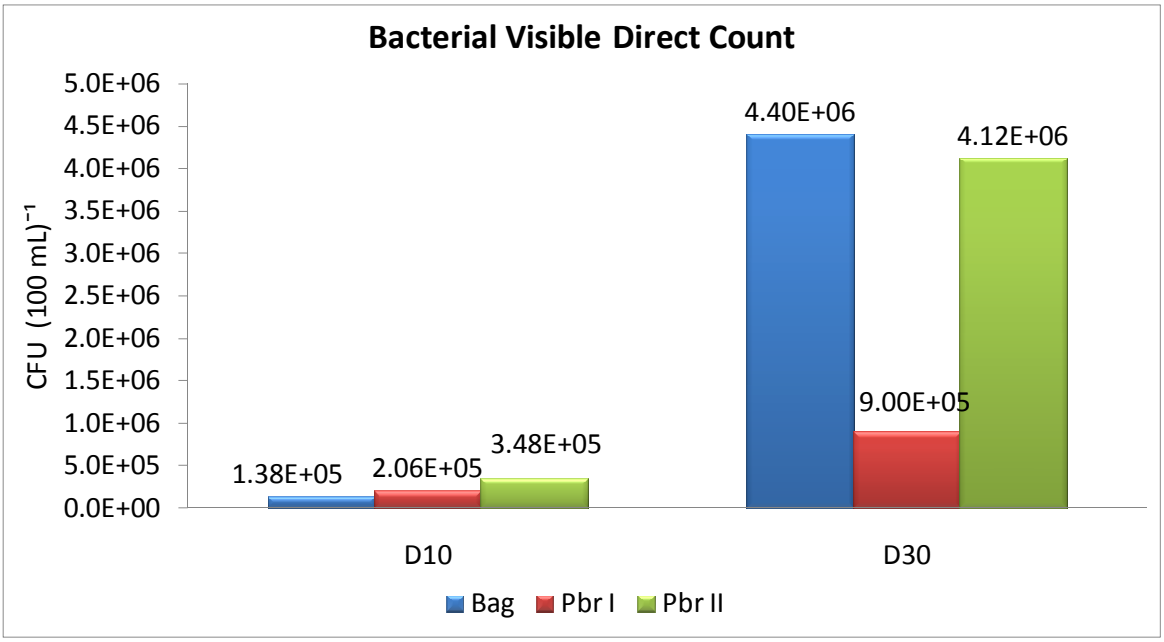


Figure 89. Bacterial visible direct count (VDC) in the three systems. Colony forming units (100(ml))$^{-1}$ on day 10 and on day 30.

The identification tests showed that yellow colonies were formed in all samples except the culture bag on day 10; these colonies were isolated in TSA (the culture bag was directly inoculated in TSA too). In all samples the bacterial colonies were gram negative (-), the oxidase and catalase test were positive (+) and all the colonies were bacilli form with positive motility presented in table VIII.

Table VIII. Identification test results on the most abundant colonies formed and isolated in the microalgae samples

	Culture system	TCBS	Gram	Oxidase	Catalase	Motility
Day 10	VPbr I	+	-	+	+	+
	VPbr II	+	-	+	+	+
	Bag	-	-	+	+	+
Day 30	VPbr I	+	-	+	+	+
	VPbr II	+	-	+	+	+
	Bag	+	-	+	+	+

The identification test **API20E™** showed codifications not reliable for the reason that they showed an ID code (4047124) that corresponds to *Vibrio alginolyticus* (Figure 90), bacterial specie that causes otitis and severe infections. This test was not done again.

Figure 90. The API20E strip ID code (4047124) of the first three samples suggested *Vibrio algynoliticus*.

4.5 Experiment four. Horizontal Photo-bioreactor (HPbr).

4.5.1 Objectives

This experiment was done to test the *N. gaditana* growth on a horizontal tubular photo-bioreactor settled outside the ICCM`s fish hatchery facilities (Figure 91) during 30 culture days and 10% daily harvest.

Figure 91. The outdoor methacrylate tubular structure of the HPbr.

4.5.2 Materials and methods

After the HPbr, was built as mentioned on the general materials and methods section, the cell concentration growth was evaluated through 30 days of culture, the initial cell concentration started at 84 x 10^6 cells $(ml)^{-1}$ with addition of nutrients at the beginning of the experiment and on the 6^{th} and 10^{th} day of culture (100% dose). The semi-continuous culture started on the 10^{th} day, with a daily harvest of 10% of the total volume replaced with the same 10% of the volume with enriched water (twice dose) through a peristaltic pump operating during daylight hours.

The system took place in a 50 L. tank with 25 liters of enriched water inoculated with five liters of previous matured *N. gaditana* culture.

Temperature was controlled by water chiller and heater installed inside the tank. The pH was controlled with CO_2 gas injection through a ph sensor inside the tank. Microalgae samples were taken on the 10^{th} and 20^{th} culture day for biochemistry analysis.

4.5.3 Results

The lag phase was imperceptible due to the exponential phase which started at day 1 reaching the late exponential phase at the 11th culture day with a cell concentration (x 10^6 cells $(ml)^{-1}$) growth of 323.75, although the daily dilution of 10% of the volume started began on day 10; the higher cell concentration of 377.5 was reached at the 15th culture day (Figure 92).

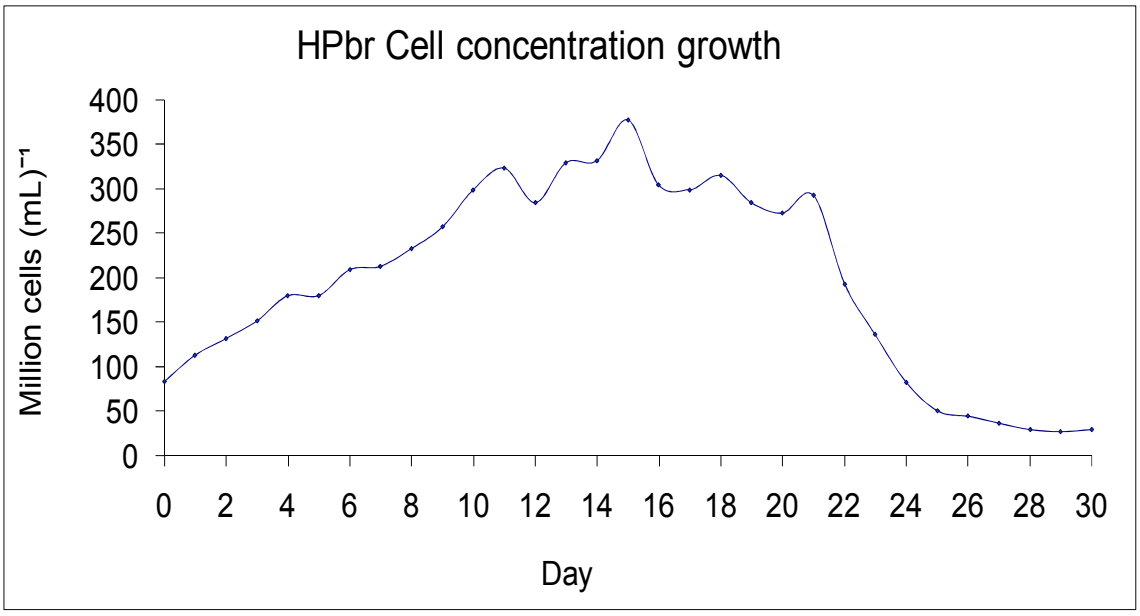


Figure 92. Cell concentration growth of *N gaditana* in the HPbr through 30 culture days.

From day 15th cell deposition in the tubular structure began to attach inside the walls avoiding the whole light entrance to the microalgae inside tubes which lead to a cell concentration decrease. This cell attachment inside the tubes continue growing covering more tube surface every day until the 22nd culture day when almost all the microalgae was attached to the tubes and other kind of algae began growing inside the tube surface (Figure 93 A, B and C) causing the culture medium water turn transparent with the lowest cell concentration of 27.5 on the 29th culture day which made impossible obtaining a microalgae sample for biochemistry analysis on the last day of the experiment.

Figure 93. Cell deposition and diatoms attached inside the methacrylate tubes. A) at the 15th culture day, B) at the 22nd culture day and C) At the lasts days of the experiment.

The microalgae daily average production (g $(L)^{-1}$) according to the 10% daily harvest was 1.31 ± 0.12 from day 10 to day 20, reaching the highest production of 1.59 on the 15th culture day as figure 94 shows. There was no more production after the 20th day due to the *N. gaditana* cell concentration decrease.

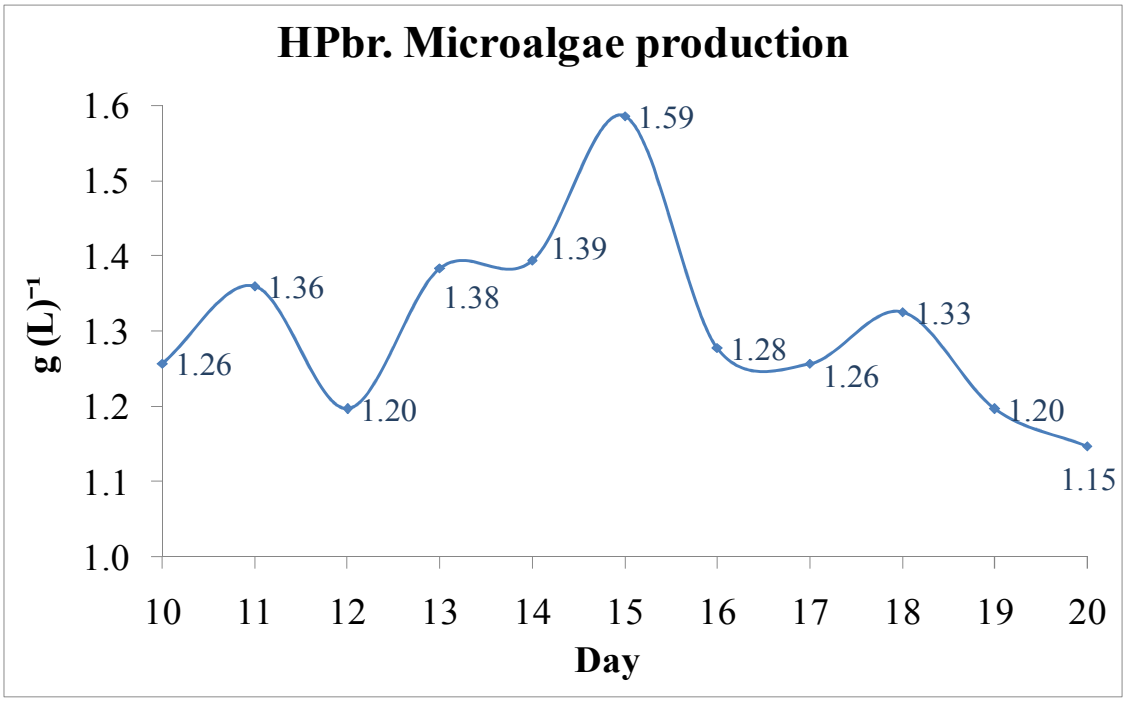


Figure 94. Daily microalgae production, 10% of the VPbr volume was harvested and replaced with the same volume of enriched water.

The water turbidity absorbance showed the cell concentration growth on the first 15 days with highest value (FAU) of 2280 on the 14th culture day, also showed the cell decrease when the walls of the tubes began to get covered by bio-fowling inside them, the water turbidity absorbance decreased to 148 in the 29th culture day (Figure 95).

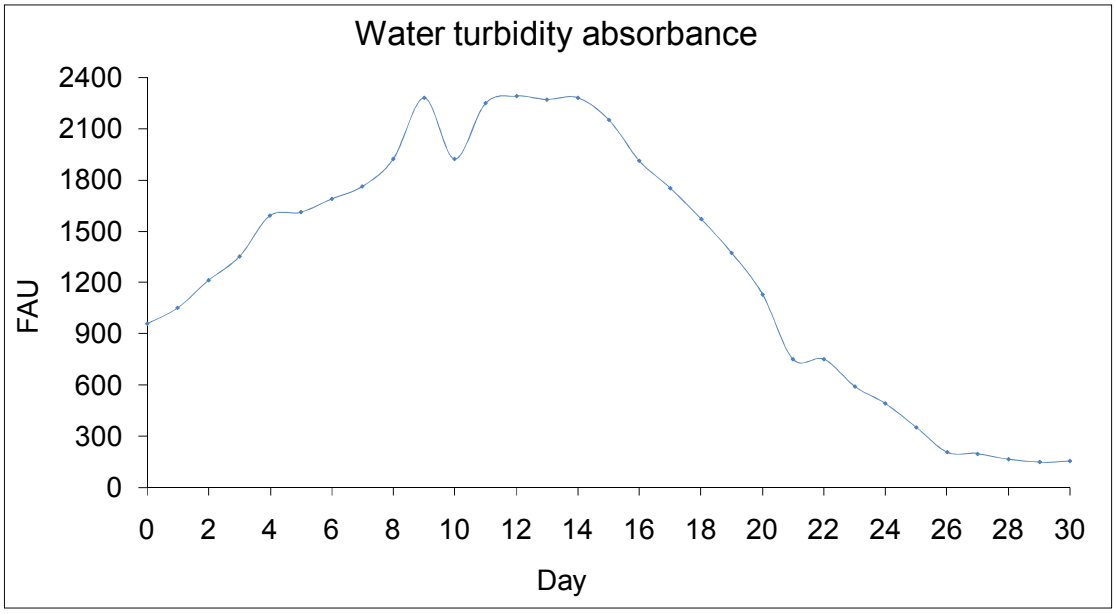


Figure 95. Water turbidity absorbance through 30 days of culture in the HPbr.

The correlation between the cell concentration and water turbidity was R^2=0.734 (Figure 96) it reflects that the cell deposition inside the HPbr tubes affected this correlation mainly in the last 15 days of the microalgae culture when the cell concentration precipitated to the lowest values.

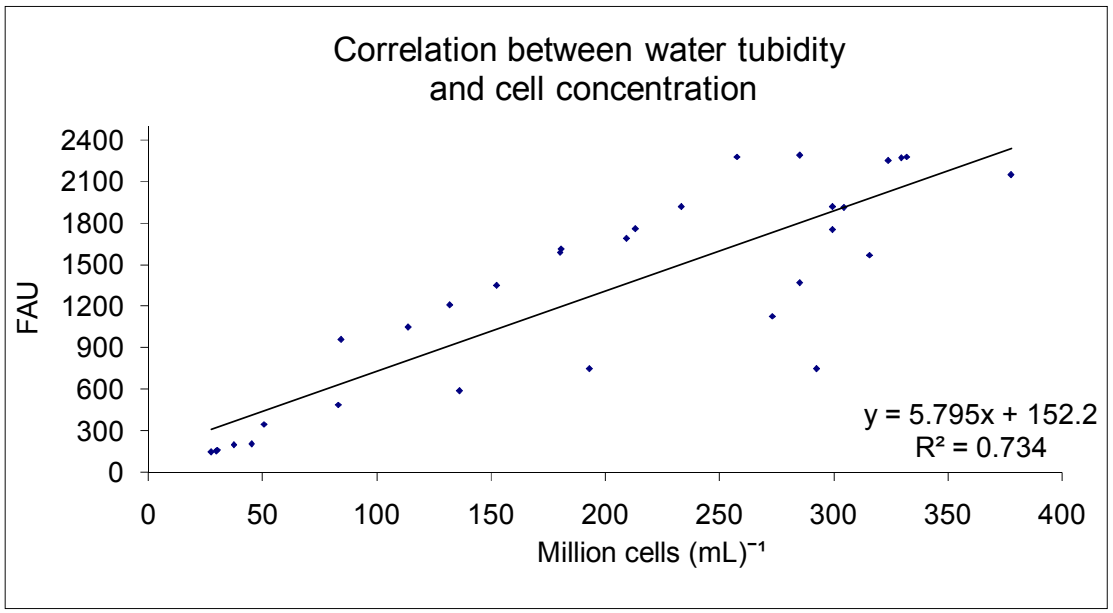


Figure 96. Water turbidity and cell concentration correlation through 30 days of microalgae.

The pH measures began on 7.7 gradually decreasing to 7.1 on day 10, after this day the effect of CO_2 gas income appeared taking the pH up again to remain near 7.5; every time the pH decreased the same patter occurred showing an oscillating behavior the last 20 days of culture (Figure 97).

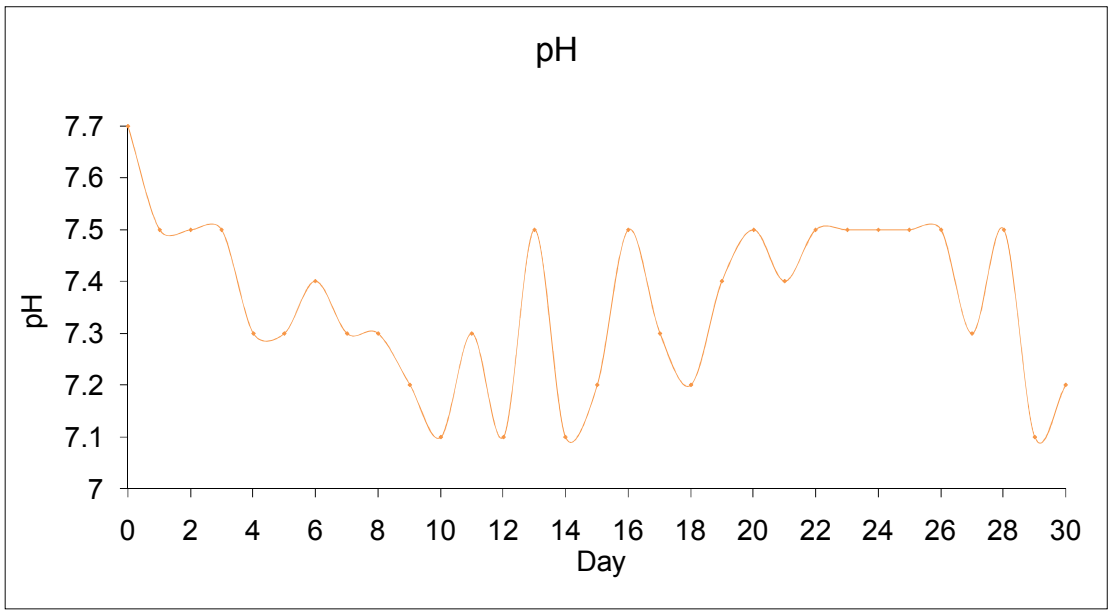


Figure 97. The pH behavior through 30 days of culture.

Temperature in the microalgae culture was controlled to remain constant. The low temperature (ºC) average was 22.29 ± 0.588 and the high temperature average was 25.4 ± 0.84. Even though the water chiller and heater, the highest temperature recorded was of 27 and the lowest 21, that is 2 ºC above and 4 ºC below the optimum of 25 ºC (Figure 98).

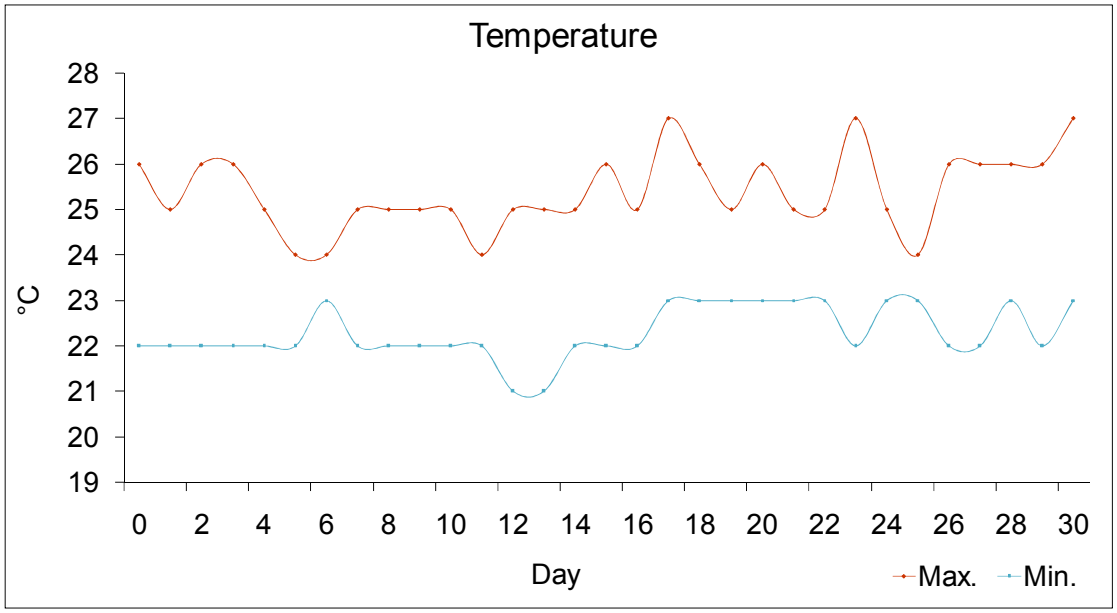


Figure 98. HPbr daily highest and lowest temperature record.

Dissolved nitrate (mg $(L)^{-1}$) gradually decreased from day 0 from 112.6 to day 6 with 51.1 when nutrients (twice dose) were added again, recovering up to 112 on day 8. After the dilutions of 10% began on the 10^{th} day the dissolved nitrate remain stable around 111.6 ± 13.22 until day 17 (Figure 99); it was when cell deposition inside the methacrylate tube encouraged other kind of algae growing attached to the tube walls and consume gradually the nutrients of the enriched water that was daily added with the dilutions despite *N. gaditana* cell concentration drastically decrease.

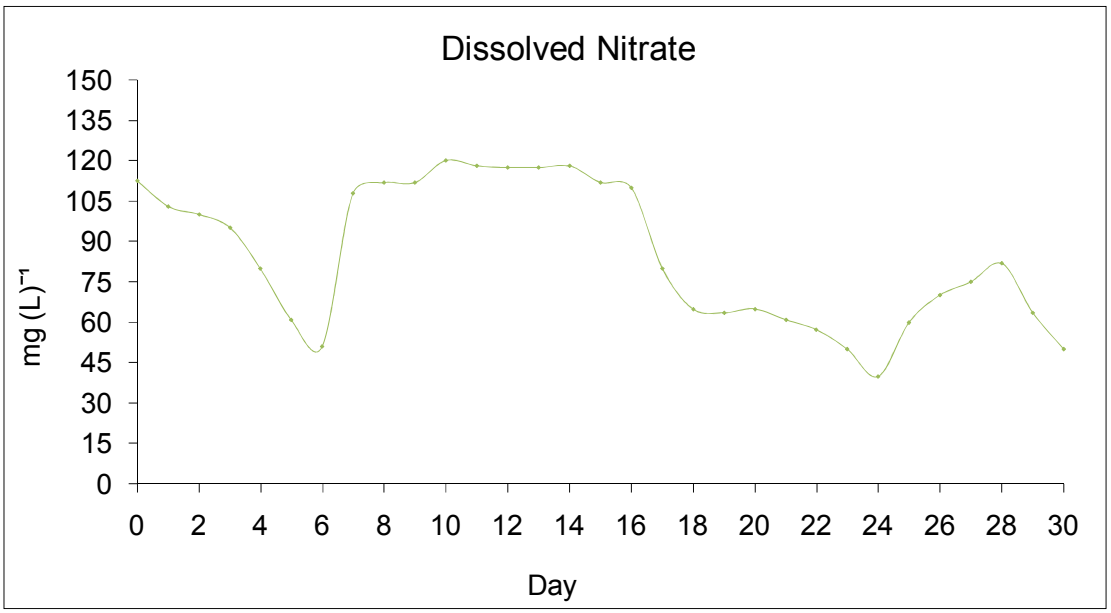


Figure 99. Dissolved nitrate during 30 culture days.

The light intensity (lux) incidence on the outside methacrylate tubes of the HPbr was 77126 ± 20564, the highest light intensity recorded was 96300 on day 1, in contrast with the lowest value of 27110 on day 6 which was a very cloudy day (Figure 100).

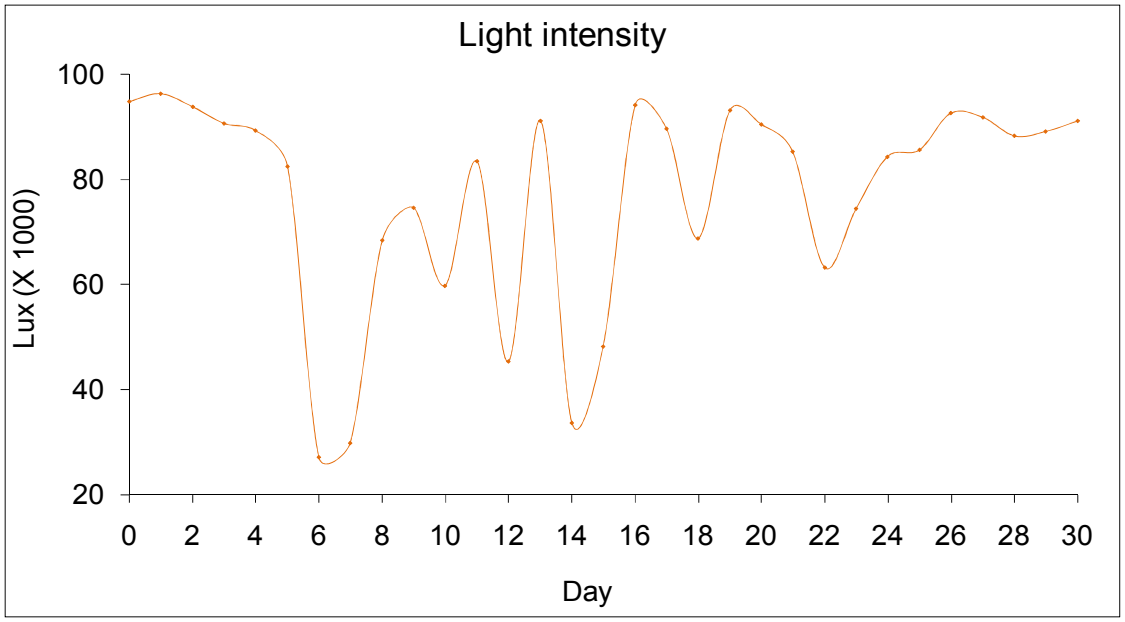


Figure 100. Light intensity variations through 30 culture days captured by the methacrylate tubes of the HPbr.

4.5.4 Biochemistry results

The moisture content (%) in the samples increased from 76.76 on day 10 to 83.8 on day 20, the same behavior occurred with the carbohydrate content which increased from 26.33 to 34.23 on the same days. The protein content decreased from 57.7 to 49.7, ashes also decreased from 4.56 to 4.38 on the same days and the lipid content maintain constant with 11.4 on the same days (Figure 101). The sample of day 30 couldn't be taken due the cell concentration decrease after the diatom growth on the methacrylate tubes.

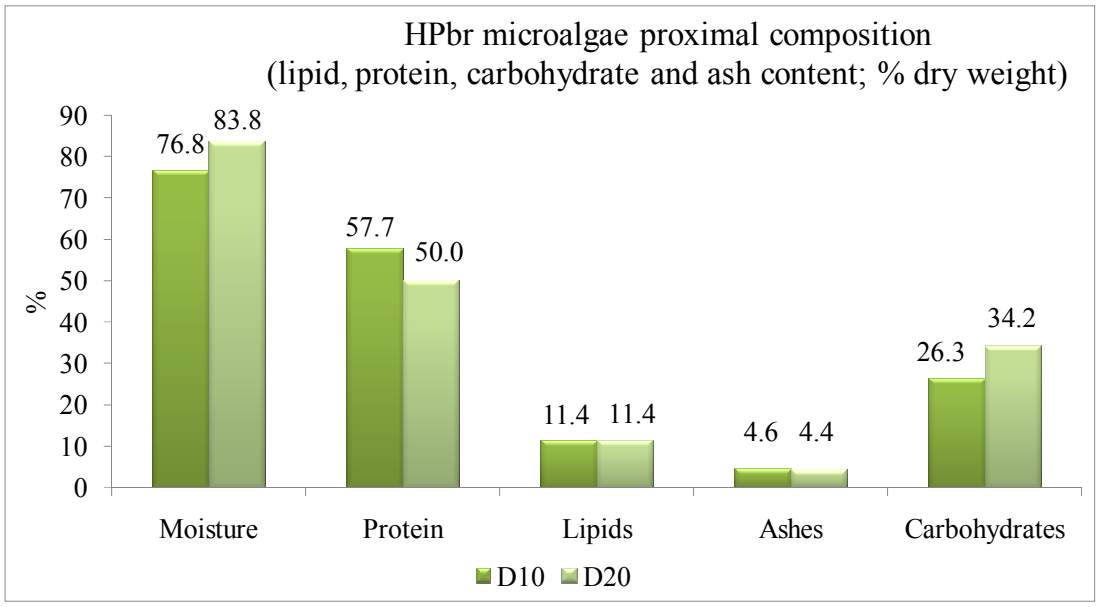


Figure 101. HPbr microalgae proximal composition on day 10 and day 20.

The microalgae fatty acids composition of *N. gaditana* produced on the HPbr is reported on table IX, values are presented in % of dry weight. Saturated fatty acids were higher in the 20th culture day with 3.590 than in the 10th culture day which was 1.349; the same proportion happened with the mono-unsaturated fatty acids which were 2.489 in the 10th culture day and increased to 3.128 in the 20th culture day. Oleic acid had a similar pattern increasing its content from 0.327 on 10th culture day to 0.348 on the 20th culture day. Figure 102 shows that total n-3 HUFA was significant higher (P>0.05) in day 10 with 6.124 than in day 20 which was 3.564; almost all the n-3 HUFA content was given by the EPA content which was 6.103 in the 10th culture day and 3.503 in the 20th culture day. ARA was higher too in the 10th

culture day with 0.556 and 0.298 in the 20th culture day. In the same proportion than in previous experiments DHA was the lowest of all n-3 HUFA`s, with 0.013 on the 10th culture day and 0.026 on the 20th culture day as it can be observed in figure 102. As the EPA content was higher in the 10th culture day the relation between DHA and EPA (Table IX) was higher in the 20th culture day with 0.007 while it was 0.002 in the 10th culture day when the EPA content was higher. In the relation case of n-3 and n-6 fatty acids, this relation was a little bit bigger in the 10th culture day with 4.97 compared with the 20th culture day which was 4.688 as shown in table IX.

Table IX. Fatty acid evaluation of the HPbr microalgae production on day 10 and day 20

Fatty acids	Day 10	Day 20
∑ **Saturated**[14]	1.349	3.590
∑ **Mono-unsaturated**[2]	2.489	3.128
∑ **n-3**[3]	6.233	3.748
∑ **n-6**[4]	1.254	0.800
∑ **n-9**[5]	0.346	0.448
∑ **n-3HUFA**[6]	6.124	3.564
14:00	0.375	0.694
16:00	0.862	2.718
16:1 n-7	1.771	2.412
18:00	0.034	0.062
18:1 n-9	0.327	0.348
18:1 n-7	0.327	0.070
18:2 n-6	0.332	0.349
18:3 n-3	0.022	0.163
20:1 n-9	0.009	0.008
ARA	0.556	0.298
EPA	6.103	3.503
DHA	0.013	0.026
DPA (22:5n-6)	0.050	0.000
DHA/22:5 n-6	0.254	0.000
EPA/ARA	10.969	11.738
ARA/EPA	0.091	0.085
DHA/EPA	0.002	0.007
DHA/ARA	0.023	0.088
oleic/DHA	25.624	13.255
oleic/n-3HUFA	0.053	0.098
n-3/n-6	4.970	4.688

[14] (1) Includes 12:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 22:0 and 24:0; (2) Includes 14:1n-5, 14:1n-7, 16:1n-9, 16:1n-7, 16:1n-5, 18:1n-9, 18:1n-7, 18:1n-5, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-11, 22:1n-9 and 22:1n-7; (3) Includes 16:2n-3, 16:3n-3, 16:4n-3, 18:3n-3, 18:4n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3; (4) Includes 16:2n-6, 18:2n-6, 18:3n-6,18:4n-6, 20:2n-6, 20:3n-6, 20:4n-6, 20:5n-6, 22:3n-6, 22:4n-6 and 22:5n-6; (5) Includes 16:1n-9, 18:1n-9, 18:2n-9, 18:3n-9, 20:1n-9,20:2n-9, 20:3n-9 and 22:1n-9; (6) Includes 20:3n-3, 20:4n-3, 20:5n-3, 22:4n-3, 22:5n-3 and 22:6n-3.

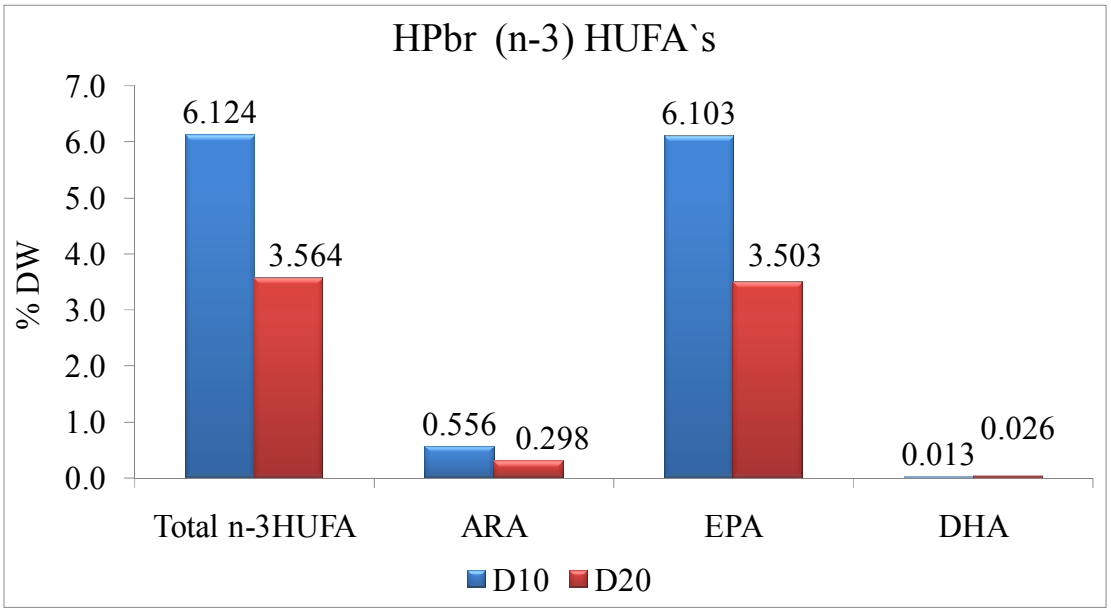


Figure 22. (n-3) HUFA`s content in the HPbr from culture day 10 to culture day 20.

5. Discussions

5. Discussions

The experiments reported in this work were carried out in order to improve *N. gaditana* production. Indeed the number of variables that may affect cell production and quality is huge with direct, indirect and cross-effect influences (Rocha, 2003). For that reason the optimization of algae production is always a difficult task considering all the algal species.

Improving protocols for finfish larval production implies reaching the equilibrium between quality of the preys and time optimization of the routine methods. One of the most time consuming step is the evaluation of algal cell concentration. Cell direct counting is a relative precise method but time consuming. A turbidimetric method, like the optical density, is very practical and easy to use (Roo, 2009; Hanhua and Kunshan, 2006). However, absorbance being related to the amount of matter also depends on the size and shape of suspended particles, their opacity, wavelength of incident light, etc (Rocha, 2003). Besides the difficulties associated to cell counting, the relationship between cell number and optical density is very dependent on culture conditions, namely the illumination, culture media, CO_2 supply, cell age, etc. In fact, the size and weight of each cell is not always the same.

A calibration curve of cell number versus optical density was done for *N. gaditana* in these trials finding a good correlation in every case. These correlations can be used for the cell growth estimations of the culture bags and photo-bioreactors in daily hatchery duties.

In the first experiment the addition of an extra carbon source was tested in order to improve algal concentration and chemical composition in plastic bags.

Hanhua and Kunshan (2006), Rocha *et al*., (2008) and Roncarati (2004), showed that adding organic (glucose) or inorganic (CO_2) carbon sources dramatically enhanced the lipid production (18.36%), EPA and n-3 HUFAs (18.24%). In this trial, the effect of addition of an extra carbon source did not enhance nor lipids (10.92%), EPA (2.69%) or n-3 HUFA (2.98%) concentrations. Some authors (Hoshida *et al.,* 2005 and Hyung *et al.*, 2009) suggest that

adding carbon dioxide to algal culture, even though more practical in economical terms, may have the disadvantage of being loss due to out-gassing processes. Anyway, CO_2 solubility depends also on pH values which influences the aqueous equilibriums (more acidic is the water, less will be the solubility of the gas). Considering this, out-gassing processes have to be excluded in both cultures (supplemented or not supplemented with carbon dioxide) because of the pH values around 8 and 9 respectively, which ensured high solubilization of CO_2. Hoshida et al., 2005 showed that a shift in pH does not affect EPA production in *Nannochloropsis sp.* Therefore, other factors (such as lack of particular nutrients, temperature, salinity or light intensity and duration) may be limiting. Temperature average (25ºC) was in the range proposed by Hanhua and Kunshan (2006) for this species which was from 14ºC to 30ºC; as well salinity (25ppm) in accordance with the range proposed by Hanhua and Kunshan (2006) from 22 to 49 ppm; but James et al., in 1998 showed that the optimal growth range for mesophilic algae is between 15-20 ºC.

The commonly used f/2 culture medium for *Nannochloropsis sp*. is supposed to be limiting for carbon and nitrogen sources (Rocha, 2003). Indeed, the medium is not supplemented with carbon considering that autotrophic organisms are able to use the atmospheric CO_2, but the addition of proportional quantities of urea (as a source of nitrogen) and glucose (as carbon source) showed an enhancement of cell growth.

Other authors (Molina *et al.*, 1999) suggested that, more than nutrients, light availability and intensity have to be considered as cardinal factors that control the productivity of photosynthetic cultures.

To overcome biases due to these previous cited factors, a second experiment in which nutrients were manipulated, was carried out.

In the second trial, nutrients manipulation was achieved both by adding the double of the regular nutrient dose and by harvesting a fixed quantity of culture replacing it with fresh

enriched water. This last approach was suggested by Miron *et al.*, 2002 and Henriques and Rocha in 2007 that already used this technique to improve algae production in their procedures. These last authors suggest dilutions varying from 10 to 30% to obtain the best productivities. Indeed, differences in cell concentration were found only when partial dilutions started (from day 10 onwards) with higher performances defined by cultures where fresh fertilized water was added. Moreover, it was found out that the best dilution rates were different if applied to cultures added with CO_2 (15%) or without CO_2 (10%). Content in EPA was influenced in an inverse manner to the dilution, because before the dilution procedure, EPA content was significantly higher ($P>0.05$) in cultures not supplemented with CO_2, while after dilution (on day 20) no differences have been detected between the treatments and almost remained stables around 5% (dw). Henriques and Rocha in 2007 indicates that harvesting a fixed quantity of culture lead to a growth pseudo-steady state, which may be a partial explanation for the stability of EPA production, in contrast with (Shifrin and Chisholm, 1981) who stated that green algae normally show an apparent enhancement in the storage of lipids due to nitrogen deficiency conditions. Also Hanhua and Kunshan (2006) showed a higher relationship between EPA accumulation (above 3%) and algae stress, in this case with both, low and high nitrogen concentration in cultures enriched with CO_2. In this experiment EPA showed always a higher content (5% vs. 3%) in respect of Hanhua and Kunshan (2006), but it must be considered that nutrients never became limiting due to the daily dilutions and replacement with enriched water.

Fatty acids profiles were almost in line with the once reported by Hanhua and Kunshan (2006) and Rebolloso-Fuentes et al., (2001); even though EPA always occupied a higher proportion of total fatty acids in respect of the previous cited studies.

Without the addition of CO_2, fatty acids showed always a higher concentration of 16:00 (5.8% dw), followed by EPA (5.4% dw) and palmitoleic acid (5.25%) regardless algae age.

On the contrary the addition of CO_2, showed that in time 16:1n-7 increased its concentration in respect to 16:00 and EPA. Hanhua and Kunshan (2006) did not notice any change in dominance of one particular fatty acid and without variation due to treatments.

It must be pointed that algae without CO_2 addition slightly increased their content in EPA with age (culture days), while decreasing their content in DHA, trend that could not be evidenced in CO_2 enriched algae; considering that N and P were probably not limiting (due to 2 times nutrient income and daily harvest), an imbalance in the C:N or C:P ratio could have been occurred, and for due to a lower carbon concentration the algae rather stored it as EPA than DHA as all ready reported for this specie (Vazhappilly and Chen, 1998).

In any case the highest cell concentration values achieved in bags was of about 50 $x10^6$ cells $(ml)^{-1}$, values that are far above for facing the needs of a hatchery principally in terms of space (Cheng-Wu, *et al*., 2001). Therefore the set up of photo-bioreactors was proposed and evaluated taking advantage of the optimized parameters of the trials at smaller scale (traditional culture bags).

The preliminary trials with vertical photo-bioreactors, confirmed the higher performance, in terms of cell growth, already explained in previous studies (Sukenik, *et al*., 2009). Indeed, the difference in cell growth was in the order of six times higher in respect to traditional culture bags. Moreover, taking advantage of the previous trials, Pbrs were started fertilizing water with twice the nutrients and adding CO_2 or applying water recirculation.

As already said, cell concentration increased dramatically since two days after the inoculum, but pollution with *Cyanobacterium sp.* may have masked the real algal growth and the effects of the different treatments. Ugwu (2008) suggested that with closed Pbrs contamination can be easily prevented due to the high algal production, but that equally, if the inoculum is polluted or, the air is not filtered, the growth of undesirable organisms may be enhanced too.

Considering the high grow rate of algae in the vertical Pbrs, a comparative experiment in which nutrients were doubled or increased four times and with a harvesting rate of 10% was carried out comparatively with a traditional culture bag. As expected, cell concentration was order of magnitude higher in Pbrs (≈ 295 ($x10^6$ cells ml^{-1})) than in bag (≈ 65($x10^6$ cells ml^{-1})), even though the same phases of growth could be evidenced in both systems, with a shorter lag phase in Pbr.

In the culture bag, microalgae decreased its lipid content in 32% from day 10 to dat 30, while doubling nutrient´s concentration (from 2X to 4X) in Pbrs, enhanced both cell´s growth and in parallel total lipids (1.4 % in Pbr with 2X nutrients *vs* 12.35 % in the 4X nutrient Pbr) and EPA production at 30 days with a general increase in the three systems (30% in the bag, 64% in 4X PBR and 4% in 2X PBR), but it was not in terms of magnitude. Miron *et al*., 2002 also had cell concentration improvement in photo-bioreactors with twice nutrient dose to avoid any nutrient deficiency. Therefore, economically speaking, it seems that the best solution in terms of cost/benefits may be the use of Pbrs fertilized with the double dose of nutrients and a daily harvesting rate of 10%.

Anyway, it must to be considered that, photo-bioreactors technology is wide with huge types of designs and performances that have been optimized during the years and adapted for different environmental conditions (Xu *et al*., 2009).

The forth experiment in this work tried to compare the performances of vertical and a horizontal Pbrs. Comparisons showed that cell growth in the horizontal photo-bioreactor was extremely more rapid than the one in VPbrs reaching after only 10 days the same cell concentration of VPbrs after 30 days (≈ 300 $x10^6$ cells $(ml)^{-1}$). Anyway, biomass was lower than the one of VPbr probably due to the smaller dimension of the algal cells. Moreover it has to be pointed out that algae from HPbr showed a lighter color than algae from VPbrs. It has been reported (Henriques and Rocha, 2007) that the production of chlorophyll a and

carotenoids increase during light period and in particular in the first 8 hours of illumination. The HPbr of this study was set up during winter time, and the recirculation of water implied that algae passed in an obscure tank almost 2/3 of the time, factor that may have influenced this behavior. Regarding lipids content, total lipids content did not differ between the treatments considering the values at the same algal cell concentration, but EPA constituted a much higher percentage of total lipids in HPbr than in VPbr (53% of TL in the HPbr, 37% and 26% in 4X VPbr and 2X HPbr respectively). Normally, microalgae improve their oil content when subjected to stressful conditions, in particular for lack of nutrients (Csavina, 2008; Hanhua and Kunshan, 2006), but contemporary, lack of nutrients implies also ceasing of cell division. In this case lowering in cell concentration was not found, indicating a correct nutrients provision, so the increase of EPA should have other explanations.

Considering overall results of the two types of photo-bioreactors, HPbr showed a very good performance both in terms of cell grow rate and lipid content, even though no extreme consideration can be done, as algae cultivation with this system collapsed after 15 days due to technical factors. Anyway, considering EPA content cells cultivated in HPbr, it must to consider the effects of the use of these algae for larval rearing and rotifers enrichment. Indeed rotifers are much prone to accumulate EPA than DHA, which may be increased if high levels of EPA are present in rotifer´s first feed, and may affect negatively larval growth (Rodriguez *et al*., 1998).

The total numbers of bacteria in the *Nannochloropsis sp*. cultures examined in this study ranged 1.38 (x 10^5) to 4.4 (x 10^6) as shown in figure 89; these values were generally in the same range of bacterial counts in coastal sea waters (from 2.78 (x 10^5) to 5.75 (x 10^6) cells/ml) according to Nakase and Eguchi, 2007. The samples were streaked in TCBS and in most of them grew positive yellow colonies; also all the isolated colonies were gram negative (-) which were red bacteria due to their thick peptidoglycan layer on their external wall, this

extensive group of gram negative bacilli form includes *Escherichia coli, Salmonella*, and other E*nterobacteriaceae*, *Pseudomonas,* α-*proteobacteria* and many others. The oxidase test was positive (+) too in all samples because they contain cytochrome c oxidase and can therefore utilize oxygen for energy production. Typically the *Pseudomonadaceae* are oxidase positive (+). The catalase test was positive to all samples which strength the *Pseudomonadaceae* bacterium presence in the microalgae culture; at last the motility test showed us that all the bacteria in the isolated samples were positive with flagellum for motility. After this point the API 20E identification strip was used and the results were *Vibrio alginolyticus* which also is a gram negative (-) colony, oxidase, catalase and motility positive, consistent with the *V. alginolyticus* isolation carried out by Gomez-Leon *et al*., 2005; but this strain identification shouldn`t be treated as a pathogen vector for fish larvae. According to Balebona *et al.,* 1998 and Bordas *et al.,* 1996, *V. alginolyticus* should be considered a pathogen for seabream only when the mucus layer in the fish gut is removed and the skin is damaged (deficient fish health) and the interaction of several environmental factors as water temperature and salinity being the most important.

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6. Conclusions

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From the present work the following general conclusions can be drawn:

1. Adding pure CO_2 or adding a double dose of nutrients to small scale algae culture (traditional plastic bags) did not improve significantly algae growth or their biochemical performance. A particular attention must to be paid on the illumination (light intensity) that seems to be a more limiting factor to algae growth.
2. Culture bags under batch conditions proved that the use of a semi-continuous system collapsed them after 10 days of harvest with any dilution rates.
3. The use of outdoor photo-bioreactors (Pbrs) dramatically enhanced algae production, considering both systems (vertical and horizontal).
4. In the onset of vertical photo-bioreactors (VPbrs) the use of a pump for water recirculation did not improve the performance of the system. Simple aeration guaranteed a good mass transfer and cell growth.
5. The addition of continuous enriched water through a peristaltic pump improved the productivity, simplifying and reducing time in daily work.
6. The microalgae productivity was enhanced at least 6 times in the Pbrs in respect to the traditional culture bags.
7. Quadruplicating nutrients (4X) for algae cultures in VPbrs, increase cell concentration, even though this difference is not in the order of magnitude with a 2X nutrients VPbr. Lipids and EPA production was also enhanced quadruplicating nutrients; in any case the advantages from quadruplicating the nutrients are not such extreme to justify the higher costs derived.
8. Horizontal Pbr (HPbr) seems to be more efficient both in terms of cell growth and EPA production in respect of VPbr, as the same cell concentration can be achieved in 1/3 of the time required for VPbr. Moreover, through the first system, concentration of

EPA on total fatty acids is a 16% more in respect of VPbr. HPbr had a better performance in terms of growth, but there are issues to be solved as the culture volume, tube size and dark phase duration for a better control of the system.

9. With VPbrs, high density and quality culture productions have been achieved, being suitable over 30 days of continuous culture reducing work time and cost. Because of this, VPbrs are widely suggested for the ICCM`s marine fish hatchery operations.

10. The bacterial load in the culture bags and photo-bioreactors is acceptable compared to coastal waters, filtering the air could improve an axenic microalgae culture; qualitative protocols for bacterial strains identification should be realized more often to record the bacterial loads that could be transferred to the fish larvae.

11. The use of Pbrs simplifies daily routine work and it also represents an advantage in terms of space since daily microalgae production required by the ICCM´s hatchery can be made in a 25% of the space that culture bag need, besides they don’t need artificial illumination and they don´t have plastic waste.

7. References

7. References

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