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Long-term dinoflagellate culture performance in a commercial photobioreactor: *Amphidinium carterae* case

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Abstract

The aim of this work was to study the culture performance of a dinoflagellate in a commercial photobioreactor. The results obtained during this long-term experiment allow to confirm that *Amphidinium carterae* is a promising dinoflagellate that can be exploited succesfully in closed systems, in semi-continuous mode in indoor and outdoor environments. The average results in an indoor 5 cm light-path 320 L photobioreactor were, in terms of specific growth rate (0.29 d^{-1}), duplication time (3.1 d^{-1}) and dry biomass productivity (78 mg L^{-1} d^{-1}). Specific compounds production was found including ω3 and ω6 fatty acids and, pigments (Peridinin, β-caroten). These promising results, besides unique characteristics found during the exploitation period such as resistance to mechanical stress, self-control of contaminant organisms, and quick cells aggregation when the culture is not in turbulence conditions, makes *A. carterae* one of the new target species suitable for commercially exploitation on an industrial scale.

Keywords

Amphidinium carterae, Dinoflagellates, Photobioreactor, Metabolites.

1. Introduction

Microalgae biomass production is one of the fastest growing biotechnology fields in recent years (Lam and Lee, 2012; Wijffels et al., 2013). This rise in the knowledge gained of microalgae biomass production in various controlled systems is supported by the increased industrial use of the metabolites obtained from microalgae. These include: essential fatty acids such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), arachidonic acid (AA), pigments (carotenoids, phycobiliproteins etc.), proteins, exopolysaccharides (EPS), and neutral lipids for biofuel production (Fuentes-Grünewald et al., 2015; Slade and Bauen, 2013; Vanthoor-Koopmans et al., 2013) All of these metabolites that can be extracted from microalgae biomass have a variety of applications from human and animal nutrition, pharmaceutical, cosmetic, and wound healing uses to use as a source of energy, amongst others.

This fast growth in microalgal production biotechnology is due to the development and improvement of the systems used for controlled production, especially closed systems like photobioreactors (PBR) (Gallardo Rodríguez et al., 2010; García Camacho et al., 2011; Zijffers et al., 2008). Microalgae biomass production has been focused on well known strains, where it is known in detail their life cycle, biotic and abiotic parameters, productivity parameters, etc. Among the most well-known microalgae species currently produced in a higher scale are: *Arthrospira maxima* and *Spirulina platensis* (both providing proteins for human consumption), *Haematoccocus pluvialis* (pigments), *Dunaniella salina* (carotenoids), *Nannochloropsis oculata* (fatty acids including EPA) (Becker, 2007; Del Campo et al., 2007; Guerin et al., 2003; Olofsson et al., 2012). Recently emphasis has been put on the search for new microalgae species that allow the production of a wide range of metabolites with high economic value, and which can be exploited under a concept of “bio-factory”, utilizing many molecules that they produce intracellularly or those metabolites excreted to the medium as

possible. The high value metabolites are the first step in full utilisation of microalgal biomass. Once these compounds are extracted from the cell or medium, the remaining fraction in the biomass can used further for a cascading set of processes such as biofuel and bioenergy uses and the recovery of nutrients (e.g. phosphate and nitrogen) used that would be required for economic feasibility of microalgae production. Various approaches are being investigated towards an economically viable cultivation and production of microalgal biomass (Slade and Bauen, 2013; Vanthoor-Koopmans et al., 2013). For these aims the dinoflagellate group is of interest (Fuentes-Grünewald et al., 2013; Gallardo Rodríguez et al., 2012; García Camacho, 2007) . In non-toxic dinoflagellates it is feasible to produce a wide range of molecules with high added value, such as: fatty acids, pigments, dimethylsulphide, polyhydroxyl, macrolides, among others compounds. There are also toxic dinoflagellates (*Protoceratium reticulatum*) that have been cultured for toxins production in controlled indoor systems. (Fuentes-Grünewald et al., 2009; Gallardo Rodríguez et al., 2010; Gallardo Rodríguez et al., 2012; Huang et al., 2009; Kobayashi and Tsuda, 2004).

Amphidinium carterae is one of the target dinoflagellate that can be exploited in closed controlled systems likes PBR, due basically to their resistance to changes in abiotic culture condition. Their production at laboratory scale has focused on understand the function of the photosynthetic apparatus in this microalgae group (Damjanović et al., 2000; Di Valentin et al., 2009) or as a producer of anti-cancer (amphidinolide) or anti-mycotic compounds (Huang et al., 2009; Kobayashi and Tsuda, 2004; Samarakoon et al., 2013). Nonetheless, despite being considered one of the easiest dinoflagellates for growing in controlled systems, their exploitation has been performed at laboratory scale and as a batch culture during short time periods (days) (Kitaya, 2008; Vazhappilly and Chen, 1998; Zimmermann Leigh, 2006). As far as we know, there is no basic information available of this dinoflagellate in terms of stable

long-term (> 8 months) culture production in commercial PBR's, in order to determine the productive parameters achievable under controlled conditions.

The aim of the present work is to give information regarding *A. carterae* production in controlled conditions, in a closed system (PBR), in a large scale (> 500 L), long-term culture (> 8 months) grown in a semi-continuous mode.

2. Material & Methods

2.1 Culture conditions

The dinoflagellate *Amphidinium carterae* ACRN03 was originally isolated from the Mediterranean Sea in the Barcelona area and was kept in the algae bank of the Institut de Ciencies del Mar (ICM-CSIC), Barcelona, Spain. For this work *A. carterae* was grown in Bio Fuel Systems S.A. facilities located in Alicante, Spain. Several previous non-axenic *A. carterae* cultures were grown in small round flasks (6 L) in controlled temperature rooms (± 20 °C), then, an inoculum was transferred first to an indoor airlift bubble column PBR with a 10 cm light-path and a total working volume of 540 L, and comprising three columns connected to a central tube collector which mixed the culture prior to sampling. The same PBR design was used for other treatments. Later on, the cultures were transferred to an airlift bubble column PBR with a shorter light-path (5 cm) and with a working volume of 320 L. Cultures were also run in an airlift bubble column PBR in outdoor conditions with a light-path of 5 cm, but with a small working volume (48 L). Detailed PBR configuration can be found in the following link: http://www.google.com/patents/US20110195493

The semi-continuous culture was performed for a period of 234 days in total, starting on 18/09/2012 and finishing on 17/05/2013. The cultures in indoor controlled conditions had light with a photoperiod of 18:6 (light:dark), an average light irradiance of 158± 22 μE m^2 s^{-1}, was measured at the external tube surface, using a Walz, ULM-500 ligth meter. An average

water temperature of 23.3± 2.2 °C and a pH of 7.7± 0.3 were recorded in indoor conditions. Non-axenic culture of *A. carterae* were grown using L1 medium (Guillard, 1995). The medium composition was: $NaNO_3$, 880 µM; $NaH_2PO_4 \cdot 2H_2O$, 36.3 µM; $Na_2EDTA \cdot 2H_2O$, 11.7 µM; $FeCl_3 \cdot 6H_2O$, 11.7 µM; $CuSO_4 \cdot 5H_2O$,0.01 µM; $Na_2MoO4 \cdot 2H_2O$, 0.09 µM; $ZnSO_4 \cdot 7H_2O$,0.08 µM; $CoCl_2 \cdot 6H_2O$, 0.05 µM; $MnCl_2 \cdot 4H_2O$, 0.9 µM; H_2SeO_3, 0.01 µM; $NiSO_4 \cdot 6H_2O$, 0.01 µM; Na_3VO_4,0.01 µM; and K_2CrO_4, 0.001 µM. In order to run in nutrient depletion mode the culture was run without any nutrient addition between day 109 and 120.

Outdoor cultures were inoculated in a small PBR (described previously), using strain from the 10 cm light-path PBR. The outdoor cultures were run in the industrial Bio Fuel Systems facilities in San Vicente del Raspeig (38° 22' 32.05'' N; 0° 32' 47.54'' W), Alicante, Spain. This outdoor culture was run for a period of 140 days, starting on 14/11/2012 and finishing on 04/04/2013. The average outdoor irradiance for Alicante in that period was 2.43 Kwh m^2 d^{-1}.

All PBR's were continuously aerated with a mixture of compressed atmospheric air and CO_2 with an average carbon dioxide of 2.5% vol/vol. The air/CO_2 flow rate was fixed at 6.25 L min^{-1}. For the study fresh filtered seawater (35 IU salinity) was used, having been previously treated with sodium hypochlorite (4 ppm) in order to avoid any contamination. Before the initial inoculation and during successive harvests (app. 30% of the total PBR volume by each harvest was chosen due to down stream process reasons) and inoculations (filled up with fresh pre-treated seawater), the pre-treated sea water was neutralized using 1mL sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$) per 1 L of sea water to be treated. The main biotic and abiotic parameters were recorded on a daily basis (week days), at the beginning of the day (09:00) for the duration of the experiment.

2.2 Cultures parameters

In order to do all the biotic and abiotic measurements during the experiment, a sample was taken from the PBR's and the following parameters were recorded immediatly. As the three bubble columns were linked and functioned as one system, a single sample was taken at each time point for all experiments.

2.2.1 Temperature and pH

Both parameters were recorded using a CRISON pH-meter model Basic 20+.

2.2.2 Absorbance (720 nm)

To determine the culture growth optical density measured as absorbance was used as a proxi of the dry weight biomass. Previously, a full absorbance scan was conducted in order to determine the specific wavelength where no interference with *A. carterae* pigments was recorded. The chosen absorbance was 720 nm, and was recorded using a Fisher Scientific Spectrophotometer model Genesys 20.

2.2.3 Dry weight (DW)

Different volumes (10, 20 or 40 mL) depending on the cell concentration in the culture, were filtered using a Whatman GF/F filter (0.7 μm, 47 mm diameter). The filters were preweighed using an analytical scale (Gram Precision ST 220S, 0.0001 g). After sample filtration, the filtered sample was washed three time with distilled water (25 mL) each time, in order to discharge any salt in the sample. Subsequently, the filters were dried in an oven (Nahita Drying 631/6) at 80°C for 24 hours. Finally, the dried filters (in duplicate) were weighed and the difference from the preweighed filter was recorded as dry weight.

2.2.4 Growth rate (μ) and Duplication time (DT)

The specific growth rate (μ), was estimated during the exponential phase of the culture. μ can be calculated as the slope of the linear regression or by the following equation:

$$\mu = \mathrm{Ln}\,(N_2/N_1)/(t_2 - t_1) \qquad \text{(Equation 1)}$$

Where LN is the natural logarithm, N_1 and N_2 are the measured absorbance at 720 nm at the initial time (t_1) and the final time (t_2).

The duplication time (DT) was defined as the time (days) that the culture needs to double their cell concentration, and was calculated by the following equation:

$$DT = LN(2)/\mu \qquad \text{(Equation 2)}$$

Where LN is the natural logarithm and μ is the specific growth rate obtained from equation 1.

2.2.5 Biomass productivity

The theoretical biomass productivity in the *A. carterae* semi-continuous culture was determined as the product between the specific growth rate (μ) obtained from equation 1 and the dry weight (DW). The real productivity was calculated as the difference in terms of DW between the sample day and the previous day. Both results are expressed in g L^{-1} d^{-1}. The theoretical productivity is only applicable to cultivation under similar conditions to those reported here.

2.3 Fatty acids analysis

Fatty acid methyl esters (FAMEs) were prepared by acid catalyzed direct transesterification and analyzed by gas chromatography as previously described by Fuentes-Grünewald et al. (2012).

2.4 Pigments analysis

30 mg of wet biomass was weighed in an amber vial and add 3370 µL of methanol added. The sample was agitated via vortexing for 15 min. After agitation the vial was incubated in darkness at 4°C for 1 hour, with one agitation by vortexer after 30 min. Subsequently the sample was centrifuged (10000 RPM for 5 min). 1 mL of the methanolic extract was taken

and was filtered through a PTFE 0.22 µm filter. Finally, the sample was injected in a HPLC (Agilent, model 1260 Infinity). The identification and quantification of the pigment concentration were conducted by comparison of their retention time, and integrating the areas under the curves with known pigments used as standards. It is likely than less than 100% of the pigments will be extracted using this method.

2.5 Elemental analysis

50 mL of culture sample was centrifuged at 10000 RPM for 5 min, the supernatant was discarded and the tube was re-filled with distilled water for washing purposes to remove excess salt and centrifuged twice. Subsequently the sample was placed in an oven (Nahita Drying 631/6) at 80°C for overnight. The day after, the sample (between 0.4 to 1.2 mg) was placed in a pre-weighed aluminium paper, then the sample was injected into an elemental analyser, CHN EuroEA. Acetanilide (C= 71.09%; N=10.36%; H= 6.71%) was used for the calibration curve.

2.6 Contamination Monitoring Program

A contamination monitoring program (CMP) was performed on a weekly basis, with the culture samples taken every Wednesday. The CMP gave a general overview of the culture in terms of contamination levels. The method includes a qualitative scale where 0 = clean (no contaminant organisms); 1 = rare (1< x < 10 organisms in one transect); 2 = scarce (11< x < 50 organisms in one transect); 3 = abundant (51< x <100 organisms in one transect); 4 = very abundant (> 100 organisms in one transect). The organisms were divided into three groups: competitors (photosynthetic organisms), predators, and cell consortia (agreggates). To conduct the identification of the contaminant species an inverted microscope (Olympus, model CKX41) was used, along with a Sedgewick-Rafter chamber, that took 1 mL of live

sample and was diluted, normally 1:10 (depending on the cell concentration). Two transects were conducted for each sample.

2.7 Statistical analysis

Data analysis was conducted using the R software, using a two factor analysis of variance allowing us to assess the differences between each treatment. When a significant difference was found, a post hoc Tukey test was used. The interaction between factors was not assessed.

3. Results and discussion

3.1 Culture conditions

3.1.1 Growth and biomass production

A. carterae cultures in the 540 L PBR indoor controlled conditions started in semi-continuous exploitation mode at day 53 (full exponential phase), and was subsequently harvested (30% of the volume) and inoculated (using pre-treated seawater) every 7 days on average. An immediate acclimation period was observed when the cultures were transferred from 6 L round flasks to the indoor PBR, due to the cultures having been transferred during exponential phase and in similar abiotic condition such as light, temperature and pH (Figure 1a). The outdoor cultures showed a longer acclimation period of 6 days on average to start growing and reached full stable growth 44 days after the first inoculation (Figure 1b). This longer acclimation period can be explained by the temperature amplitude (discussed later) and the higher light irradiation founded in outdoor conditions in the Alicante area (2.43 Kwh m^2 d^{-1}).

Regarding the growth measured as optical density (720 nm), it showed no significant statistical difference (p=0.204) among the PBR's depending on the light-path. The maximum

average absorbance value reached in small scale 6 L round flasks was 0.667±0.3. In relation exclusively to the PBR's, the maximum absorbance value reached by the indoor 10 cm light-path PBR was 0.690 with an average of 0.459±0.1. In contrast the PBR with a shorter light-path (5 cm) indoor had an average absorbance of 0.781±0.1, a 170% higher than the 10 cm light-path indoor PBR.

Regarding the outdoor PBR with a 5 cm light-path, the absorbance peak recorded was 0.747 and the average value was 0.323±0.1. The maximum absorbance value was higher than the 10 cm indoor PBR but, considerably lower than the same light-path reactor in indoor conditions (Figure 1a & 1b).

The absorbance values recorded in the PBR's in indoor and outdoor conditions coincide with the growth rate (μ), biomass productivity and duplication time (DT) that is shown in table 1. The growth rate, biomass productivity and the duplication time are higher in the 5 cm light-path PBR than the 10 cm light-path PBR. These parameters improved when the culture was transferred to the 5 cm light-path PBR starting at day 170, a higher growth rate (> 152%), higher productivity (>159%) and an average of 1 day less in terms of DT were obtained in this PBR (Figure 1a and table 1). The highest absorbance value recorded in the 5 cm light-path PBR in indoor condition coincides with the highest cell concentration recorded ($4.4x10^6$ cell mL^{-1}) and the maximum biomass production (0.496 g L^{-1}) reached by *A. carterae* during the whole experiment.

In respect of biomass productivity, it is clear that the average and the maximum productivities achieved during the experiment were those recorded in the 5 cm light-path indoor PBR starting at day 170, with the productivity peaking at day 181 with a value of 125 mg L^{-1} day^{-1} (Figure 2).

When the average results in terms of biotic parameters for the indoor condition PBR against outdoor condition PBR were compared, the statistical ANOVA analysis showed a significant

impact of both factors (light-path and indoor-outdoor condition) on productivity, with significant differences between the values in each treatment (light path: p=0.03; indoor-outdoor condition: p=0.01). The outdoor PBR showed -31% lower biomass productivity, -39% in growth rate and on average took 2.28 days more to double their biomass. Independently of the light-path in the reactors, the outdoor PBR had lower biotic values. The main biotic parameter in microalgae cultures (μ) was considerably higher in the indoor PBR with the same light-path, reaching 2.2 time more growth in controlled conditions. (Table 1). For those culture (in indoor and outdoor conditions) that grew with a wider light-path (10 cm), their growth rates were similar to those obtained by Zimmermann 2006, although the cell concentration obtained during our experiment were one order of magnitude higher than the Zimmermann work. When it was compare the difference in terms of growth rate and the main biotic parameters such as biomass productivity, cell concentration and dry weight between the two light-path PBRs that have been studied, is clear that a shorter light-path (5 cm) significantly improves these biotic parameters (Figure 1a and table 1). In fact, the growth rate obtained by different authors (Ho et al., 2003; Kitaya, 2008) are coincident with those obtained in the present work, however, it was obtained a higher cell concentration (one order of magnitudes) than the work of Franklin & Berges 2004, or even two fold magnitudes more than the Kitaya's works. These improvements in the biotic parameters can be explained by the higher light availability in the shorter light-path PBR used in our experiment, or by the efficiency on the light absorption of the peridinin-chlorophyll-protein (PCP) light-harvesting complex exhibit by dinoflagellates like *A. carterae* where energy transfer efficiencies up to 95% have been found, implying a greater capacity to receive and process this light energy (Damjanović et al., 2000; Polívka et al., 2006).

3.1.2 Abiotic parameters

Concerning the main abiotic parameters measured during the trial, the temperature recorded in indoor controlled conditions had an average of 23.3±2.1°C with a peak of 29.4°C and a minimum of 18.4°C. Although the temperature was controlled in the indoor PBR, a seasonal fluctuation was observed, with lower temperatures recorded between day 68 to 172 of culture, coinciding with the winter season in the northern hemisphere (Figure 1c).

In the case of the outdoor PBR, the average temperature recorded was 22.5±4.2°C with a peak of 30.0°C and a minimum of 11.0°C, showing a higher amplitude temperature and fluctuation among days, these differences and temperature fluctuations can be explained by no temperature control in the outdoor PBR, the cloud cover, and the natural difference between night and day in outdoor conditions in the Alicante area.

Concerning pH, this abiotic parameter showed a similar evolution in both cultures conditions, independent of the light-path in the PBR. The pH in indoor controlled conditions was 7.7±0.3 with a maximum of 8.65 and a minimum of 6.97. The outdoor PBR showed a slightly lower average pH 7.27±0.4, with a peak of 9.05 and a minimum of 6.63 (figure 1d). Both cultures showed the lowest pH value at the beginning of the day and no carbonate precipitation was found inside the PBR when higher pH values (> pH 8.5) were recorded.

These *A. carterae* abiotic cultures conditions were similar results found to Kitaya et al., 2008, Samarakoon et al., 2013 and Zimmermann 2006 in terms of optimal temperature, finding that the best temperature for growing conditions should be between 20°C to 25°C. However, it is noted like other dinoflagellates a temperature amplitude higher than 10°C lengthens the lag phase or conditioning time of the cultures, especially in those exposed to outdoor conditions (Fuentes-Grünewald, 2012). It would be highly recommended to perform a Life Cycle Assessment (LCA) prior to deploy an industrial outdoor dinoflagellate culture, to elucidate if

this lower growth is compensated by the lower production cost (by using natural light, no temperature control etc.).

The pH showed maximum nocturnal-diurnal difference of 1.5 unit, but always the pH values were slightly neutral-acidic. Those slightly acidic values were due to the use of CO_2 in order to keep the cultures with carbon dioxide constantly available, and it seems to be that *A. carterae* likes these conditions because as in the work of Kitaya et al., 2008 when there is an increase in CO_2 the growth performance in the culture is better. It must be noted that when the pH values were higher than 8.5 unit neither a calcium carbonate formation nor a slow-down of the culture in terms growth rate were observed.

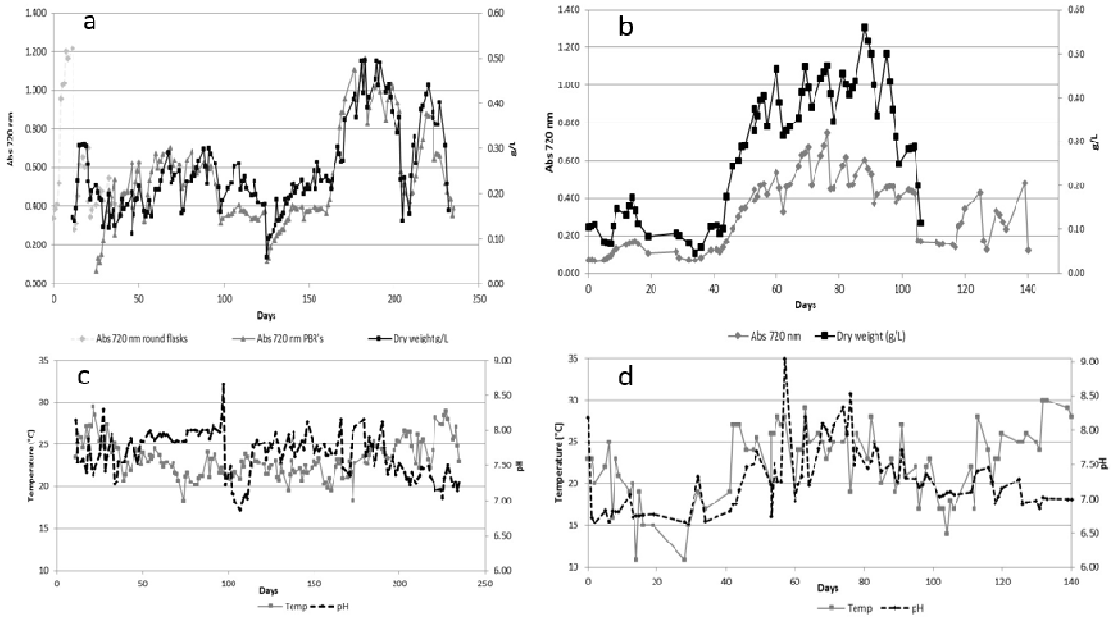

Figure 1. Growth curve in indoor condition (1a) and outdoor condition (1b) recorded as a dry weight (black line) and optical density using absorbance at 720 nm (grey line). Main abiotic parameters in indoor (1c) and outdoor condition (1d) temperature °C (grey line) and pH (dash black line).

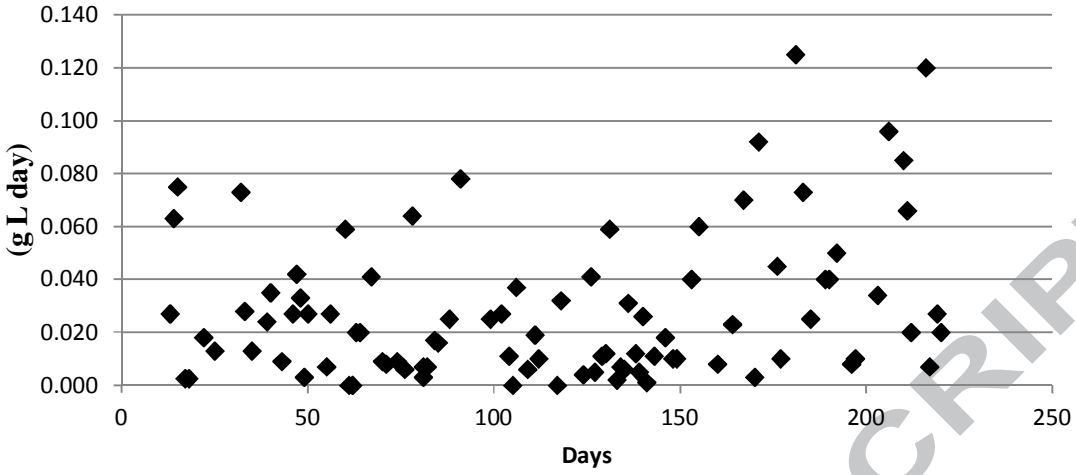

Figure 2. Biomass productivity in *A. carterae* cultures in indoor PBR's. Productivity of the 10 cm light-path from day 13 to day 170, 5 cm light-path from day 170 to day 223.

Table 1. Averages of the main biotic parameters recorded during the growth of *A. carterae* in controlled indoor condition and outdoor condition. L-P: Light-Path. Two factor ANOVA test (L-P and in-outdoor condition) on productivity was conducted (*: $p<0.05$, Df: Degrees of Freedom). Tukey: post hoc test comparing the different factor levels.

Biotic parameters	Outdoor PBR 48 L L-P 5 cm	Indoor PBR 540L L-P 10 cm	Indoor PBR 320L L-P 5 cm	Indoor average
μ (day^{-1})	0.13±0.05	0.19±0.06	0.29±0.16	0.21±0.11
Duplication time (days)	5.77±2.06	4.10±1.36	3.10±1.69	3.49±1.50
Real biomass productivity (g L day^{-1})	0.036±0.02	0.049±0.02	0.078±0.02	0.052±0.03
Theoretical biomass productivity (g L day^{-1})	0.039±0.02	0.038±0.01	0.087±0.03	0.049±0.03

3.2 Fatty acids

The main free fatty acids in *A. carterae* identified during the experimental period are shown in Table 2. The highest percentages in the fatty acid profile are those of the saturated group (SAFA), with on average above 80% in this fraction. Among the saturated, the highest and predominant one was the palmitic acid (C16:0) with an average of 76.91%, followed far behind by the miristic acid with an average of 5.1%. The second group of fatty acids detected were the unsaturated fatty acids (USFA) with an average of 10%, the majority being palmitoleic acid C16:1 (6.5%) and then oleic acid C18:1 (4.0%). The polyunsaturated fatty acids (PUFA) were the lowest percentage group among the different fatty acid groups with an average of 2% of the total fatty acids. DHA was the highest one in this group with an average of 1.52% of the total fatty acids and with peaks of 3.95% at day 109 (Table 2). Among the PUFA's the second one is the EPA with an average of 0.52% and a peak of 1.07% at day 109. Note that unusually the boost in the PUFA fraction and especially in those high value fatty acids as DHA and EPA were recorded when the culture was run in nutrient depletion mode (between day 109 and 120) in the indoor PBR.

Table 2. Methylated fatty acid profile (%), extracted on different days from *A. carterae* PBR indoor semi-continuous cultures

Fatty acids	Day 53	Day 68	Day 81	Day 89	Day 109	Day 120	Day 177	Day 184	Day 203
C14:0	6,13	3,25	6,11	4,69	3,01	4,90	9,60	4,86	4,07
C16:0	75,59	77,99	78,48	75,51	72,84	74,28	77,69	79,41	80,44
C16:1	6,04	8,61	8,53	6,01	3,45	5,80	8,54	7,57	4,45
C18:1	2,17	3,81	2,26	4,57	8,44	8,71	1,66	2,39	2,55
C18:2	1,07	0,56	0,44	1,08	2,74	2,15	0,47	0,98	0,67
C18:3	0,00	0,00	0,00	0,00	0,07	0,07	0,00	0,00	0,00
C20:0	6,60	3,82	4,12	6,16	4,44	0,69	2,03	3,37	5,72
C20:5*n*3	1,03	0,39	0,00	0,39	1,07	0,81	0,00	0,52	0,47
C22:6*n*3	1,37	1,58	0,07	1,58	3,95	2,59	0,00	0,90	1,64
Saturated	88.32	85.06	88.71	86.36	80.29	79.87	89.32	87.64	90.23
Unsaturated	8.21	12.42	10.79	10.58	11.89	14.51	10.20	9.96	7.00
Polyunsaturated	3.47	2.52	0.51	3.05	7.82	5.62	0.47	2.41	2.78

A high concentration of saturated fatty acids (SAFA) were shown when the culture condition are nutritionally optimal, this can be explained because most of this SAFA are used by photosynthetic cells mainly during the growth process, being an essential part of the cell membrane (in form of glyco or phospholipids) in most of the microalgae groups (Olofsson et al., 2012). When the cultures were running in nutrient stress conditions (between day 109 and 120) principally by the absence of nitrogen in the medium, it was noticed that there was an important increase in the C16:0/C16:1 - EPA/DHA ratios, and a reduction in the C:N ratio (table 4). These results are coincident with the results obtained in dinoflagellates in previous studies (Fuentes-Grunewald et al., 2012) and, even in a different microalgae groups such as the Chlorophyceae, Prasinophyceae among others (Ho et al., 2003). All these variations can be explained by the metabolic change exhibited by microalgae cells when they are submitted to nitrogen depleted conditions. It is well known that the nitrogen deficiency in the culture triggers an enhancement of the triacylglicerides lipids not just in dinoflagellates, but also in

most of the microalgae groups (Converti et al., 2009; Fuentes-Grünewald et al., 2013; Li et al., 2008; Olofsson et al., 2012). Strangely *A. carterae* showed an increase in the PUFA fraction rather than in tha SAFA when is submitted to nutrient stress.

Regarding specific high value fatty acids production in *A. carterae* cultures, there was an interesting production of these metabolites, especially the docosahexaenoic (DHA). Actually, if we compare the maximum percentage of DHA in the PUFA fraction found in our *A. carterae* samples (3.95 %), they were more than double of those obtained by Vazhappilly & Chen 1998. It should be note that the maximum EPA and DHA content in *A. carterae* samples were achieved when the culture were run in depleted nitrogen condition. It is well know that in depleted nutrient condition (especially nitrogen) the amount of lipids in different microalgae groups such as chlorophyceas or dinoflagellates increase drastically (Fuentes-Grunewald et al., 2012; Li et al., 2008), in our case the cultures shown the same pattern. Although, there is scarce information regarding the production of essential fatty acid (ω3 y ω6) in this species, it can be indicated that our maximum production during this study (3.6 mg L^{-1} for DHA and 1.1 mg L^{-1} for EPA) was lower than those obtained by Vazhappilly & Chen 1998, probably because our cultures were runned in autotrophic mode. The higher EPA and DHA concentration obtained in Vazhappilly & Chen 1998 work can be explained by the use of high acetate or glucose concentration used as a carbon source, and because they grew *A. carterae* in heterotrophic mode.

3.3 Pigments

The pigments found in *A. carteare* samples from indoor cultures are shown in table 3. It should be pointed out that the amount of the carotenoid pigment peridinin predominated with an average of 4.25 mg g^{-1} of dry biomass, followed distantly by chlorophyll *a* with average values of 1.06 mg g^{-1} of dry biomass. Other pigments detected during the experiment were β-carotene, lutein and zeaxanthin, all quantified in small concentrations (< 0.1 mg g^{-1} of dry

biomass). It was noticed that the amount of the main pigments such as chlorophyll and peridinin that are actually part of the peridinin-chlorophyll-protein (PCP) light-harvesting complex in dinoflagellates, were always detected in higher concentration when the culture entered into nutrient depleted conditions, and this condition (nutrient depletion) also cause a discoloration in the culture, turning from a healthy maroon to a pale brown (personal communication).

Table 3. Main pigments (mg/g of dry biomass) extracted in different days from *A. carterae* PBR indoor semi-continuous cultures.

Pigments	Day 68	Day 89	Day 109	Day 120	Day 184	Day 203
Zeaxanthin	-	-	-	-	0,02	-
Lutein	0,02	-	-	-	0,02	-
Chlorophyll *a*	0,09	0,01	0,06	4,39	0,88	0,95
β-Carotene	0,08	-	-	-	0,15	0,18
Peridinin	0,35	0,06	0,22	17,55	3,53	3,81

3.4 Elemental analysis and ratios

Table 4 shows the elemental analysis results (C, N, H), alongside the different analysed ratios for lipids during the study. The main elements found in *A. carterae* samples was carbon, average of 49.2±2.9 %, and then an average of 9.5±3.3 % for nitrogen and 6.8±0.4 % for hydrogen. When the evolution of the C:N ratio is analysed a reduction can be seen when there is an increase in the intracellular nitrogen content, and the lowest values in the C:N ratio coincide with the nutrient depletion mode in the indoor PBR. This nutrient depletion mode

also triggered the increase in the PUFA fraction and a rise in the ratio of the main fatty acids C16:0/C16:1. In the same nutrient depleted period there was an increase in the EPA:DHA ratio due to the increase in the DHA content.

Table 4. Elemental analysis, C16:C18, EPA:DHA, and C:N ratios in *A.carterae* samples extracted during the experiment in a semi-continuous mode indoor PBR

Parameters	Day 53	Day 68	Day 81	Day 89	Day 109	Day 120	Day 177	Day 184	Day 203
% N	10,1	4,5	10,2	9,4	13,1	15,6	9,7	8,4	4,9
% C	51,2	52,2	51,5	44,2	49,0	52,2	44,7	47,8	49,7
% H	7,2	7,5	6,9	6,1	6,6	7,0	6,7	7,0	6,5
C16:0/C16:1	12,8	9,2	9,2	12,8	22,2	13,3	9,1	10,6	18,4
EPA:DHA	0,8	0,2	0,0	0,2	0,3	0,3	0,0	0,6	0,3
C:N	5,1	11,7	5,1	4,7	3,7	3,3	4,6	5,7	10,2

3.5 Contamination Monitoring Program

The contaminant organisms detected in *A.carterae* cultures in indoor conditions, were generally competitors (photosynthetic organisms), followed by predators. Among the phototrophic organisms the highest level reached was for small cyanophyceas (level 3: abundant) and dinoflagellates from the Gymnodinium genus with a level 1: rare. In the case of the predators the only organisms found during the whole trial was a ciliates protozoa from the Paramecium genus reaching a level 2: scarce, in the qualitative scale. It should be noted

that these contamination issues were detected after moving the culture from the 10 cm L-P indoor PBR to the 5 cm L-P, and it was probably during the culture movement (using peristaltic pumps) that these organisms entered the culture. Nevertheless, it must be pointed out that *A. carterae* shows a certain self-cleaning capacity, because the contaminant organisms never reached important concentrations of competitors or predators, and the main contaminant organisms disappeared when *A.carterae* cultures reached over $2x10^6$ cell mL^{-1}.

3.6 Nutrient uptake

Other results were obtained that confirm it is feasible to grow a dinoflagellate like *A. carterae* in long term semi-continuous mode in indoor and outdoor conditions. It was noted that during the semi-continuous mode (feeding with full L1 medium) the nitrate uptake time was the same after consecutive harvest-inoculation events, showing an uptake average time of 7 days, equivalent to 5.23 µmol per hour.

3.7 Species characteristic

Is interesting noted that the performance of *A. carterae* during this exploitation regime (semi-continuous) showed suitable attributes for industrial scale culture. Despite it being an athecate dinoflagellate, it is quite tough as it withstood all the cultures transfers (normally using peristaltic pumps), with no evidence of morphological change or cell damage. Another interesting characteristic of this species is the ability to form clumpy cell aggregations when is not in a turbulent regime. It is important to highlight this peculiarity of *A. carterae* cells during the harvest time, because it showed a high sedimentation speed of 1.5 hour per meter of column, and could be used as a harvesting strategy by natural flocculation reducing processing costs.

Currently one of the major challenge in microalgal biotechnology is to find a robust algae that can be used as a biofactory to produce several expensive metabolites and that can be cultivated in semi-continuous mode for a longer period (Greenwell et al., 2010). The results

presented in this paper confirm that the dinoflagellate *Amphidinium carterae* is a good candidate for a high scale microalgal production. Basically, due to their good acclimation and long-term growth in indoor and outdoor conditions in PBR's, for their ability to produce several expensive metabolites such as DHA and EPA fatty acids, pigments (peridinin) and others compounds suggested by other authors such as macrolides (amphinolides) that can be used for cancer treatment, or long chain polyketides such as lutheophanol, colopsinol, with known antibacterial and antifungal activities (Huang et al., 2009; Kobayashi and Tsuda, 2004; Samarakoon et al., 2013).

The adaptation of *A. carterae* to outdoors conditions, is similar to those obtained in the same microalgae group but with different species (Fuentes-Grünewald, 2012; Fuentes-Grünewald et al., 2013). It did not show cell damage when the cultures were submitted to turbulent conditions compared with other species of the same microalgae group (Gallardo Rodríguez et al., 2010). *A. carterae* cultures had show a low level of competitors or predator contaminant organisms present, possibly due to the exudation of sulfuric compounds such as dimethylsulfide or the allelopathic characteristic founded in dinoflagellates when they release secondary metabolites to the medium that inhibit the growth of others organisms (Garcés et al., 2013; Legrand et al., 2003). It is also important to highlight the *A. carterae* feature of forming cellular aggregates when the culture is not in turbulent conditions, which enables a short time of sedimentation, thereby simplifying and helping to save economic and energetic resources during the harvest stage. All of these characteristics show the positive prospects of *A. carterae* culture to be successfully used in an industrial scale cultivation.

Conclusion

Amphidinium carterae is a promising dinoflagellate that can be exploited succesfully in larger scale semi-continuous culture**,** because of its versatility in terms of acclimation to closed systems such as photobioreactor in both indoor and outdoor conditions, growth rates, biomass productivity, and the number of expensive metabolites capable of being exploited. Also it is noted that the culture features found during the present work such as self-cleaning ability against contaminants, cell strength to mechanical actions, and the capacity to form cell agregations during the harvest, allow us to confirm that this dinoflagellate is a suitable candidate for microalgal biomass industrial production.

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Highlights:

- *Amphidinium carterae* is a good candidate to be exploited in larger scale
- A short light-path improves the main biotic parameters in *A. carterae* cultures
- Several high value metabolites can be extracted from *A. carterae* biomass
- Natural floculation and self cleaning properties were found in *A. carterae* cultures