Mar Biotechnol (2013) 15:37–47
DOI 10.1007/s10126-012-9450-7

ORIGINAL ARTICLE

Biomass and Lipid Production of Dinoflagellates and Raphidophytes in Indoor and Outdoor Photobioreactors

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Received: 8 September 2011 / Accepted: 11 April 2012 / Published online: 29 April 2012

Abstract The principal fatty acids from the lipid profiles of two autochthonous dinoflagellates (*Alexandrium minutum* and *Karlodinium veneficum*) and one raphidophyte (*Heterosigma akashiwo*) maintained in bubble column photobioreactors under outdoor culture conditions are described for the first time. The biomass production, lipid content and lipid productivity of these three species were determined and the results compared to those obtained when the strains were cultured indoors. Under the latter condition, the biotic values did not significantly differ among species, whereas under outdoor conditions, differences in both duplication time and fatty acids content were observed. Specifically, *A. minutum* had higher biomass productivity (0.35 $g \cdot L^{-1}$ day^{-1}), lipid productivity (80.7 mg $lipid \cdot L^{-1}$ day^{-1}) and lipid concentration (252 mg $lipid \cdot L^{-1}$) at harvest time (stationary phase) in outdoor conditions. In all three strains, the growth rate and physiological response to the light and temperature fluctuations of outdoor conditions greatly impacted the production parameters. Nonetheless, the species could be successfully grown in an outdoor photobioreactor and were of sufficient robustness to enable the establishment of long-term cultures yielding consistent biomass and lipid production.

Keywords Lipids · Microalgae · Photobioreactor · Dinoflagellates · Raphidophytes · Biodiesel

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Introduction

One of the main goals of microalgal biomass production is to find new algal strains capable of growing in enclosed systems and yielding high biomass productivity and lipid content (Griffiths and Harrison 2009; Greenwell et al. 2010). The industrial-level production of microalgal biomass is essential to the goal of replacing, at least partly, the demand for fossil fuels with more sustainable and environmentally friendly third-generation biodiesel. To date, only restricted microalgal genera mostly belonging to the Chlorophycean group such as *Chlorella vulgaris*, *Chlorella protothecoides*, *Chlamydomonas reinhardtii* and *Neochloris oleoabundans* have been used in these attempts, in experiments limited to the laboratory or pilot scale (Liang et al. 2009; Li et al. 2010; Widjaja et al. 2009). However, these laboratory–pilot observations cannot be extrapolated to outdoor conditions because the physiological behaviour is strain-dependent, and when the microalgae are subjected to natural external conditions such as light irradiance, light cycles and temperature, their physiology varies continuously as well as the culture performance.

Since the 1970s, there have been several projects in which freshwater algae were especially cultivated for biomass production (Benemann et al. 1982; Borowitzka 1988; Sheehan et al. 1998). However, the massive amounts of freshwater, already in short supply in many places throughout the world, needed for industrial-scale algal cultivation would replace one

environmental problem with another. Therefore, in line with the recommendation to build "the reactor around the algae" (Morweiser et al. 2010), the target of third-generation biodiesel production must be to exploit seawater microalgae isolated from the same areas where biomass culture and production facilities are located, working with local algae and using in situ conditions.

The biotechnological use of microalgae biomass for biofuel production has been developing rapidly over recent years (Chisti 2007; Hu et al. 2008). The target species for biomass production have traditionally been those with a known growth cycle, fast cell growth and those that usually were cultivated for other aims: as a protein source such as *Tetraselmis suecica* or the cyanobacteria *Spirulina platensis* or those microalgae used for aquaculture activities such as *Isochrysis galbana*, *Nannochloropsis oculata* (Chiu et al. 2009; Rodolfi et al. 2008) and others that produce special metabolites such as the freshwater algae *Haematoccocus pluvialis* (Zhekisheva et al. 2002; Grewe and Griehl 2008) or *Scenedesmus almeriensis* (Sanchez et al. 2008). These algae are widely used in industry in the synthesis of pigments and as food additives. Other recently described algae suitable for biomass production are those from the marine dinoflagellates and raphidophytes groups (Fuentes-Grünewald et al. 2009, 2012). Some genera from this group were successfully cultured in different culture systems such as flasks, bubble columns and photobioreactors (PBRs) (Parker et al. 2002; Fuentes-Grünewald et al. 2009; Gallardo-Rodriguez et al. 2010), but most of these works were done in small volumes under laboratory conditions. Moreover, some species of dinoflagellates and raphidophytes are known to produce toxins in controlled cultures (Gallardo-Rodriguez et al. 2010), and this ability to produce toxins can be used to obtain target commercial biomolecules (Shimizu 2003; Garcia-Camacho et al. 2007).

Another crucial aspect to bear in mind when implementing microalgal biomass facilities is the choice between the two principal culture systems currently employed in microalgal biomass production: enclosed or open systems. Enclosed systems mainly consist of PBRs in the form of a bubble column or flat panel, while open systems include open ponds or raceways (Carvalho et al. 2006; Chen et al. 2011). Each system has its advantages and disadvantages, with the final choice depending on the desired final product. Enclosed systems are more expensive than open ponds in terms of construction, maintenance and energetic requirements, but they allow the control of abiotic parameters, such as temperature, pH and CO_2 injection. Consequently, the biomass obtained is of a higher quality and has a higher volumetric concentration (Carvalho et al. 2006). Open systems have lower construction costs; they require less maintenance and have lower energy requirements, but biomass productivity is lower and there are problems related to evaporation and contamination by other microalgal species, fungi and protozoa (Benemann 2008).

Culture facilities for microalgal production are usually indoors, and the abiotic parameters are controlled to allow for efficient growth of the strains, resulting in efficient biomass, high-quality products and a constant harvest of microalgal biomass. However, this strategy is accompanied by a high energy demand and thus by high production costs (Norsker et al. 2011; Sevigné et al. 2012). By contrast, outdoor conditions involve variations in temperature and solar irradiance. Furthermore, biomass production is irregular as it depends on the geographic location and on the season of the year. The advantage of an enclosed system under outdoor conditions is the lower energy costs (Chen et al. 2011; Sevigné et al. 2012).

To date, there have been no pilot-scale assessments of the growth, biomass and lipid production of dinoflagellates and raphidophyte strains cultured outdoors. Moreover, there is little information on the growth and physiological response of microalgae exposed to the varying environmental conditions of outdoor culture systems, as well as indoor information about biomass production and lipid concentration. The aim of this study was to evaluate the growth, biomass productivity, principal fatty acid concentration and physiological responses of three species of microalgae, specifically, two dinoflagellates (*Alexandrium minutum* and *Karlodinium veneficum*) and one raphidophyte (*Heterosigma akashiwo*), when cultured in an enclosed system (a bubble column photobioreactor) under outdoor versus indoor conditions.

Materials and Methods

Photobioreactor Design

The bubble column photobioreactor (bcPBR) used in the indoor and outdoor conditions of this study is schematised in Fig. 1. The nine polymethylmethacrylate tubes (2.0 m height and 0.15 m Ø) had a volume of 0.035 m^3 each, with a total bcPBR working volume of 0.315 m^3. In the outdoor conditions, these tubes were inclined at an angle of 15 ° with respect to the incident sunlight (east–west orientation). For indoor conditions, they were vertically positioned at 0.1 m distance from the light source to avoid heating. Agitation of the column's contents was achieved by continuously injecting pre-filtered air (Iwaki filter, 0.2-μm pore size) with an air flow rate of 0.1 $v/v \times min^{-1}$. The injected atmospheric air was provided by an air pump; the average CO_2 concentration measured was 420±14 ppm (Qubit systems CO_2 analyser) with no external supply of carbon dioxide injection to the bcPBR. The air flow rate had been previously tested with respect to optimal microalgal growth and was found to achieve a well-mixed supply of nutrients while avoiding the

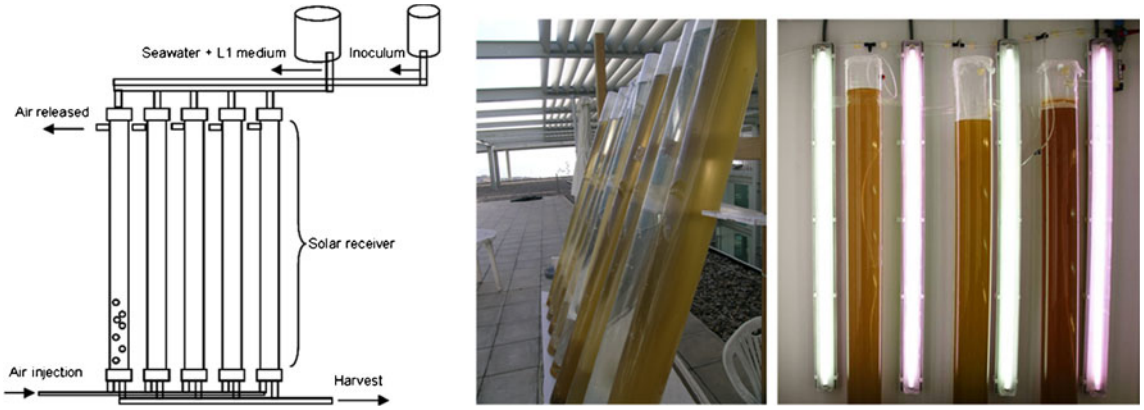

Fig. 1 Schematic bubble column photobioreactor (bcPBR) and photographs of the bubble column in the outdoor and indoor conditions

formation of reactive oxygen species (Gallardo-Rodríguez et al. 2010).

Experimental Design

Indoor and outdoor experiments were conducted at the Institute of Marine Sciences (ICM-CSIC), Barcelona, Spain (41 ° 23′ 08.12″ N–02 ° 11′ 45.84″ E). The dinoflagellates *K. veneficum* (ICMB 252) and *A. minutum* (AMP4) and the raphidophyte *H. akashiwo* (ICMB 830) were isolated from natural samples collected from the northwest Mediterranean Sea. The strains were used throughout the experiments, with cultures run in triplicate.

Both indoor and outdoor cultures were grown in filtered (0.21 μm) seawater, with a salinity of 37 and initial pH average of 7.99±0.03 (measured with Eutech Instruments PCD 650 Waterproof Portable Meter), obtained from the ICM culture facilities, supplemented with full L1-enriched seawater without added silicate (Guillard and Hargraves 1993). The composition of the medium was as follows: $NaNO_3$, 880 μM; $NaH_2PO_4{\cdot}H_2O$, 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; $Na_2MoO_4{\cdot}2H_2O$, 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.

Indoor conditions consisted of an environmentally controlled room where all abiotic parameters were constant. Microalgae cultures in the experimental bcPBR tubes were subjected to a 12:12 h light/dark (L/D) cycle. Illumination was provided by a 1:1 combination of Gyrolux fluorescence (58 W; Sylvania, Erlangen, Germany) and cool-white (58 W; Philips, Eindhoven, The Netherlands) tubes; together emitting a photon irradiance of 110 μE m^{-2} s^{-1} (measured with a Li-Cor photometer Li 185-B).

Indoor batch cultures were maintained in a temperature-controlled room at 20±1°C. Under indoor conditions, several batch cultures of the strains were done previously and the results were similar in terms of their biotic parameters (Fuentes-Grünewald et al. 2009, 2012).

For outdoor conditions, nine tubes (bcPBR) were placed on the ICM-CSIC terrace (Fig. 1). The experiment was started in mid-November and finished in mid-May (autumn–winter and spring, respectively, in the northern hemisphere). The cultures were run in semi-continuous mode; harvesting biomass was done depending on duplication time of the species. Half of the biomass in the tubes was removed by gravity for sampling, and the volume was replaced by filtered seawater and fresh new medium (Fig. 1). The arrows in Fig. 2 show the harvest time for each species. As in the indoor conditions, pre-filtered air was used for agitation inside the bubble column. The temperature inside the tubes was recorded with a temperature data logger (HOBOware) every 30 min. throughout the experiment. Solar radiation data were obtained from the Catalonia meteorological station (http://www.meteo.cat/xema), using the station nearest to our institute's location (approximately 1 km). Daily average solar radiation between 10:00 AM and 02:00 PM was calculated during the period time of the outdoor experiment and was expressed in μE m^{-2} s^{-1}. Discrete in situ measurements of photosynthetically active radiation were conducted in indoor and outdoor conditions measured with a Li-Cor photometer Li 185-B

Growth Rates and Duplication Time

Net growth rates were estimated during the exponential phases for the three strains. Every 2 or 3 days, 10 ml subsamples was withdrawn from each culture and fixed in Lugol's iodine solution. Cell abundances were estimated at×200–400 magnification using a Sedgewick-Rafter chamber and inverted optical microscopy (Leica-Leitz DM-II, Leica Microsystems GMbH, Wetzlar, Germany). Net exponential growth rates, μ(div*day^{-1}) (Guillard 1995), were calculated as the slope of the regression line of ln (N) vs. time (t), with N defined as the estimated cell

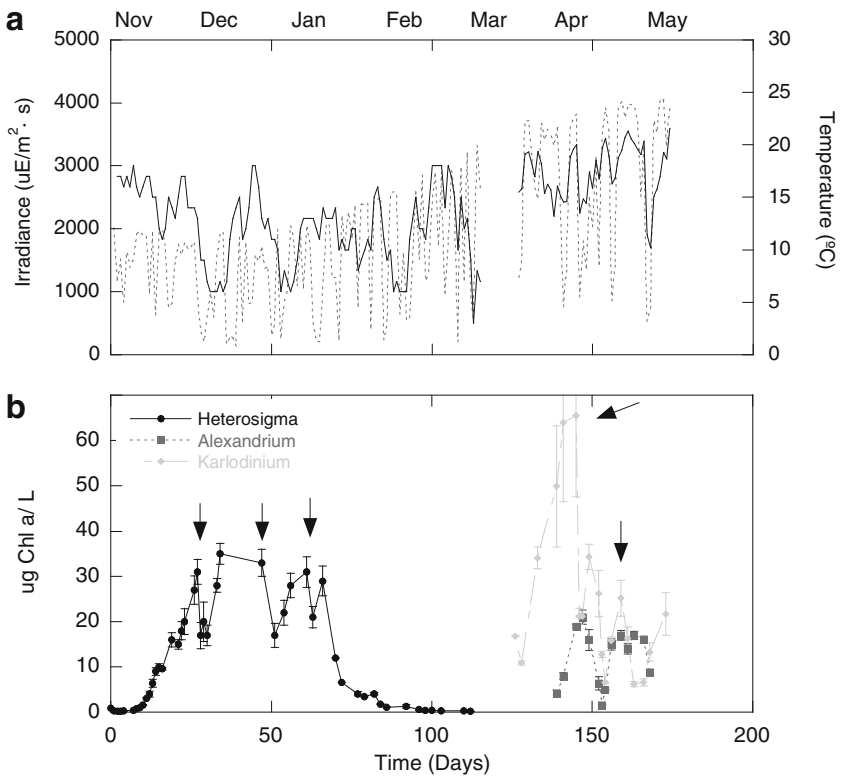

Fig. 2 Daily average of solar radiation (*dashed line*) and water temperature (*solid line*) during the study period inside the bcPBR (**a**); growth curve in outdoor conditions of the strains tested (**b**) (*arrows* indicate harvest time)

concentration or chlorophyll *a* measured in micrograms Chl *a* L^{-1} (Eq. 1).

$$\mu = \mathrm{Ln}(N_2/N_1)/(t_2 - t_1) \quad (1)$$

Where N_1 and N_2=cell number at time 1 (t_1) and time 2 (t_2), respectively.

Duplication time (T_d) was defined as the time (days) needed to double the algal concentration (Eq. 2)

$$T_d = \frac{\mathrm{Ln}\,(2)}{\mu} \quad (2)$$

To compare cell count and due to the extension of the outdoor experiment, chlorophyll *a* (as a proxy of biomass) was estimated. Ten millilitres of live samples was read directly in the fluorometer (Turner Designs Fluoremeters) and biomass expressed in micrograms Chl *a* per litre.

Principal Fatty Acids

Lipids from the three strains of microalgae were analysed twice during the growth curve: during the late exponential phase and during the stationary phase. At each phase, triplicates of 50 ml were filtered on pre-combusted (450°C 4 h) GF/F Whatman glass fibre filters, immediately frozen in liquid N_2, freeze-dried for 12 h, and then stored at −20°C until analysis (~5–10 days). The filters were placed in a tube with 3:1 dichloromethane–methanol (DCM/MeOH) spiked with an internal standard (2-octyldodecanoic acid and 5β-cholanic acid). Lipids were extracted using a microwave-assisted technique (5 min at 70°C), previously described as being simplest and most effective for microalgal lipid extraction (Kornilova and Rosell-Mele 2003; Escala et al. 2007; Gómez-Brandón et al. 2008). After centrifugation, the extract was first taken to near dryness in a vacuum centrifuge maintained at a constant temperature and then fractionated by solid-phase extraction, according to a previously published method (Ruiz et al. 2004). The sample was subsequently re-dissolved in 0.5 ml of chloroform and eluted through a 500-mg aminopropyl mini-column (Waters Sep-Pak® cartridges) previously activated with 4 ml of *n*-hexane. The first fraction (neutral lipids) was eluted with 3 ml of chloroform/2-propanol (2:1) and the fatty acids were recovered with 8.5 ml of diethyl ether/acetic acid (98:2). The free fatty acid (FFA) fraction was methylated using a 20 % solution of MeOH/BF_3 and heated at 90°C for 1 h, yielding fatty acid methyl esters (FAMEs). The reaction was quenched with 4 ml of NaCl-saturated water. FAMEs were recovered by extracting the samples twice with 3 ml of *n*-hexane. The combined extracts were taken to near dryness, re-dissolved with 1.5 ml of chloroform, eluted through a glass column filled with Na_2SO_4 (to remove residual water), and, after chloroform removal, subjected to nitrogen evaporation. The extracted sample was stored at −20°C until gas chromatography (GC) analysis. At that time, the extracts were re-dissolved in 30 μl of isooctane and then analysed in a Thermo Finnigan Trace GC Ultra instrument equipped with a flame ionisation detector and a splitless injector and

fitted with a DB-5 Agilent column (30 m length, 0.25 mm internal diameter, and 0.25 μm phase thickness). Helium was used as the carrier gas, delivered at a rate of 33 cm s^{-1}. The oven temperature was programmed to increase from 50 to 320°C at 10°C min^{-1}. Injector and detector temperatures were 300 and 320°C, respectively. FAMEs were identified by comparing their retention times with those of standard fatty acids (37 FAME compounds, Supelco® Mix C4-C24) and quantified by integrating the areas under the curves in the GC traces (Chromquest 4.1 software), using calibrations derived from internal standards.

Dry Weight Biomass

Dry weight (DW) was determined by filtering duplicate subsamples (10 ml) of the culture, through preweighed glass fibre filters (Whatman GF/F 25 mm, nominal pore size 0.7 μm). The filters were dried in an oven (105°C) for 4 h and then weighed in a Sartorious scales (precision of ±0.001 g).

Biomass Productivity, Lipid Content and Lipid Productivity

Biomass productivity in semi-continuous culture was determined as the product of the specific growth rate in the exponential phase of the culture (μ obtained from Eq. 1) and the dry weight biomass concentration (DW), with the results expressed as grams per litre per day. Lipid content was reported as a percentage of dry weight. Lipid productivity (oil produced by the algae, by volume and time) was calculated as the product of biomass productivity (in grams of DW per litre per day) and lipid content (percent of DW).

Data Analysis

To determine which biotic parameters were improved when the cultures were submitted to natural conditions of growth, data of the biotic parameters (Table 1) were transformed ($\log \times + 1$) to normalise variance for parametric analysis. The effects of treatments on the strains biotic parameters were compared using a multifactorial ANOVA with a confidence of 99 %. Specified factors for the ANOVA included conditions (outdoor or indoor) and species (*H. akashiwo*, *A. minutum* or *K. veneficum*). The Tukey's HSD test was used after ANOVA to identify significant differences between mean values, with a probability level of 1 % ($P<0.01$) indicating significance. All statistical analyses were conducted with STATISTICA© 6 software for PCs.

Results

Environmental Conditions and Abiotic Parameters

The first experiment under outdoor conditions was conducted with *H. akashiwo* during the autumn–winter. This first approach allowed us to improve culture conditions (injected air, conditioning time, tube maintenance and cleaning) in the outdoor environment and was followed by a second experiment using *A. minutum* and *K. veneficum* during the spring in the northern hemisphere (Fig. 2b).

For *H. akashiwo* cultures, irradiance and temperature in the outdoor treatment were highly variable throughout the studied period. The average temperature recorded inside the bcPBR was 12.1°C, with a minimum of 6°C in mid-December, with no culture death. However, a second minimum with temperatures near 0°C occurred in early March, corresponding to an unusual snow storm and resulting in the sudden death of the cultures (Fig. 2a). The night–day temperature amplitude during the autumn–winter study period was close to 10°C, with the minimum recorded early in the morning (around 07:00 AM) and the highest temperatures at noon. Minimum light irradiance on the *H. akashiwo* cultures was recorded at the end of December (143 μE m^{-2} s^{-1}), and the maximum at the beginning of March, 3321 μE m^{-2} s^{-1} (Fig. 2a). Irradiance and temperature inside the tubes were highly correlated ($r^2=0.87$). At high irradiance (>2,500 μE m^{-2} s^{-1}), temperatures rapidly climbed to >15°C (with a delay of <4 h between the two variables), especially in March, at the end of the growth period of *H. akashiwo*. The initial pH of the *H. akashiwo* culture in the outdoor bcPBR was 7.99±0.03, reaching a maximum of 8.95 after 28 days and with an average for the entire autumn–winter period of 8.36±0.04.

The lag phase of *H. akashiwo* in the outdoor environment was relatively long, almost 10 days (Fig. 2b), after which the strain began to grow exponentially. The stationary phase in the culture was reached between days 20 and 25, which enabled the first harvesting of *H. akashiwo* biomass (arrow, Fig. 2b). Subsequently, the strain reached the stationary phase of the growth curve within 4–6 days after the inoculation of new fresh medium and seawater (consistent with the average duplication time for *H. akashiwo*). From the beginning until the end of the experiment, *H. akashiwo* cultures were run for almost 120 days in semi-continuous mode.

K. veneficum and *A. minutum* were cultured in the spring. Accordingly, both temperature and irradiance were higher than during the period of the *H. akashiwo* culture. The average temperature for the culture period of *K. veneficum* and *A. minutum* was 17.1°C. The maximum light irradiance was 4020 μE m^{-2} s^{-1} and the minimum was 202 μE m^{-2} s^{-1} (Fig. 2a).

Table 1 Biotic parameters of the strains tested under indoor and outdoor culture conditions

	Outdoors			Indoors		
Species	*Heterosigma akashiwo*	*Alexandrium minutum*	*Karlodinium veneficum*	*Heterosigma akashiwo*	*Alexandrium minutum*	*Karlodinium veneficum*
Taxon	Raphidophyte	Dinophyta (thecate)	Dinophyta (athecate)	Raphidophyte	Dinophyta (thecate)	Dinophyta (athecate)
T_d (days)	5.32±1.4	2.66±1.1	3.63±1.6	4.56±2.3	5.87±0.8	5.45±0.6
Dry weight biomass (g L^{-1})	1.46±0.1	1.20±0.03	1.06±0.02	1.15±0.1	1.14±0.04	1.14±0.06
Biomass productivity (g L^{-1} day^{-1})	0.25±0.04	0.35±0.11	0.22±0.07	0.20±0.09	0.16±0.02	0.15±0.01
Maximum lipid content (%)	23	22	12	24	23	27
Maximum lipid concentration TAG+FFAs (mg L^{-1})	253.3	252.0	127.1	214.6	253.4	257.9
Lipid productivity (mg L^{-1} day^{-1})	56.1±9.7	80.7±23.9	26.7±8.6	48.8±14.7	36.5±4.9	40.3±3.0

Duplication time (T_d) was calculated in the exponential growth phase. Productivities were measured during the same phase of culture (harvest time) and are expressed in volumetric unit (±SD)

TAG triacylglycerides, *FFAs* free fatty acid

K. veneficum entered the late exponential growth phase around day 18 after the inoculation (Fig. 2b), reaching values of >60 μg Chl *a* L^{-1}, followed by a second growth phase in which Chl *a* concentrations were 20 to 30 μg L^{-1}. The corresponding values for *A. minutum* were similar to those of *K. veneficum* during the first exponential growth phase but were reached in less time (<10 days) in the latter. The Chl *a* values for *A. minutum* during the two exponential growth phases were between 15 and 20 μg Chl *a* L^{-1} (Fig. 2b).

Effect of Culture Conditions and Species on Biotic Parameters

In general, the main biotic production parameters (growth rate, biomass productivity as dry weight, lipid concentration and lipid productivity; Fig. 3, Table 1) were higher in outdoor than in indoor conditions (F=224.7, P<0.01) and there were significant differences between species (F=21, P<0.01) given the better results for *A. minutum*. In addition, the interaction between biotic factors was significant (F=42, P<0.01).

The growth rates of the three species under indoor conditions did not significantly differ (according to a post hoc Tukey's test after a significant ANOVA, Tukey's test P< 0.01). By contrast, under outdoor conditions, the growth rates of the three species varied widely and were greatly different over time due to the variability of the natural irradiance and the fluctuating temperatures (Fig. 3).

No significant differences (according to a post hoc Tukey's test, P<0.01) were found between the strains with respect to biomass productivity and dry weight in indoor conditions (Table 1). In outdoor conditions, the dry weight biomass of *H. akashiwo* was significantly higher than that of the other two species (Tukey's post hoc test P<0.01).

Maximum Lipid Content, Lipid Concentration and Lipid Productivity

Lipid content was similar between strains except for *K. veneficum* in outdoor condition (Table 1). Maximum lipid concentration, expressed as the sum of triacylglycerides plus the FFA fraction, yielded similar values between strains and

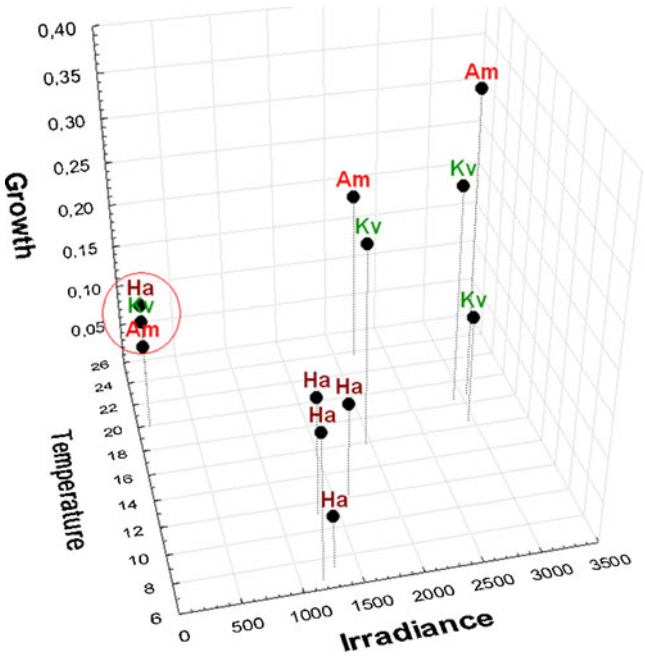

Fig. 3 3D graph showing the growth rates (div*day^{-1}) of the *Heterosigma*, *Karlodinium* and *Alexandrium* strains tested in this study under the different incident natural light (μE m^{-2} s^{-1}), and temperature (ºC) submitted to outdoors conditions. The growth rates of the strains under controlled irradiation and temperature at indoors conditions is evidenced with a *circle*

culture conditions except for *K. veneficum* outdoor conditions (Tukey's test $P<0.01$, Table 1). On a per cell basis and in outdoor conditions, the highest amount was achieved by *A. minutum* (4.40 ng lipid per cell), followed by *H. akashiwo* (4.17 ng lipid per cell) and the lowest amount was obtained by *K. veneficum* (3.55 ng lipid per cell). On a per cell basis and in indoor conditions, the maximum lipid content was recorded in *A. minutum* (4.08 ng lipid per cell), with *H. akashiwo* lagging far behind (1.37 ng lipid per cell) and followed by *K. veneficum* with 1.26 ng lipid per cell (data not shown).

In terms of lipid productivity, the best performance was *A. minutum*, which under outdoor conditions produced three times more lipids than *K. veneficum*. Indoor lipid production was not significantly different between strains (Tukey's test $P<0.01$), with *H. akashiwo* as the highest producer (Table 1).

Principal Fatty Acids

Figure 4 shows the main fatty acids recorded, divided into saturated fatty acids (SAFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). SAFA accounted for the largest proportion, specifically, palmitic acid C16:0, followed by stearic acid C18:0 and myristic acid C14:0. The second largest contributor of fatty acids was the PUFA fraction, especially eicosapentaenoic acid C20:5*n*3. The MUFA fraction was comparatively small, with palmitoleic acid C16:1 as the main contributor.

The SAFA concentration differed between the species and culture conditions. Indoor conditions produced the highest SAFA concentrations: 49.8 lipid mg L^{-1} for *K. veneficum* at culture day 20, 46.7 lipid mg L^{-1} for *H. akashiwo* at culture day 23 and 29.7 lipid mg L^{-1} for *A. minutum* also at culture day 23. MUFA were detected in only two of the three strains cultured indoors, with a maximum concentration at day 23 for *H. akashiwo* and *K. veneficum* of 3.9 and 2.7 lipid mg L^{-1}, respectively. Regarding the PUFA fraction, and specifically eicosapentaenoic acid, this high-value oil was produced by only one species, *H. akashiwo*, with larger amounts obtained from indoor rather than outdoor cultures, and the maximum of 9.8 mg L^{-1} occurring on culture day 20 (Fig. 4).

In general, the lipid profiles of strains cultured outdoors were similar to those obtained from indoor cultures, with the highest concentrations accounted for by the SAFA fraction, followed by the MUFA and PUFA fractions (data not shown). A comparison of the total concentration of FFAs resulting from indoor vs. outdoor culture conditions showed differences in *A. minutum* and in *K. veneficum* at ca. 20 days; however, there was no difference (Tukey's test $P<0.01$) in the FFAs analogously produced by *H. akashiwo* (Fig. 4).

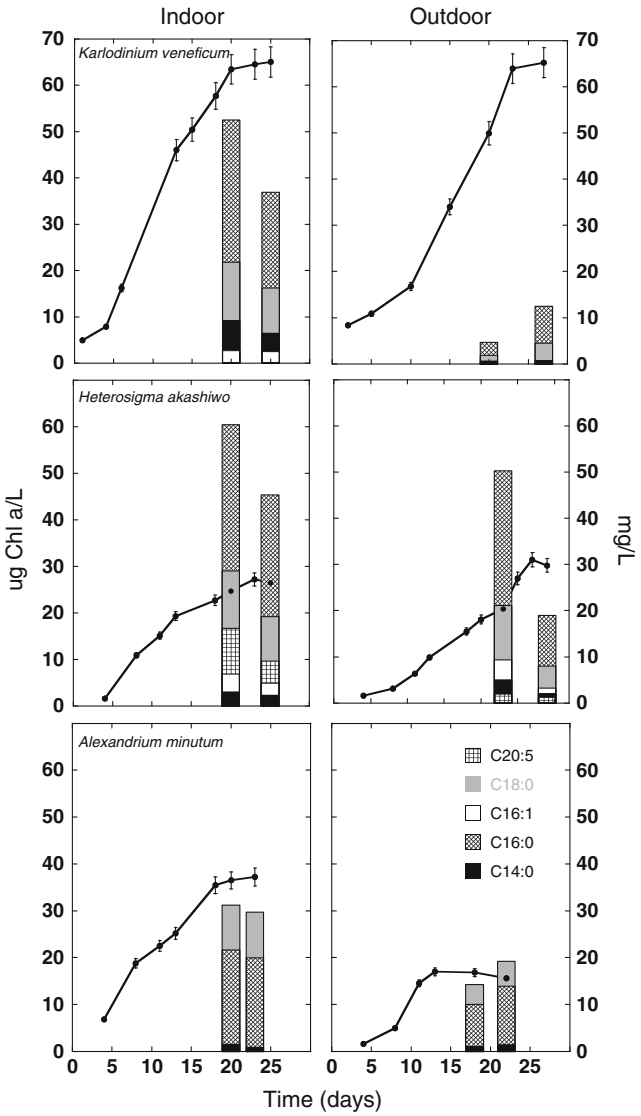

Fig. 4 Growth curve (*black line*) and fatty acids concentrations (*bars*) of the three strains cultured in bubble column photobioreactors under indoor and outdoor growth condition. Fatty acids were measured during the late exponential and stationary phase of the cultures

Discussion

The present study confirms that cultures of dinoflagellate and raphidophyte strains can be maintained over the medium term (months) under outdoor conditions in the Mediterranean area. Previous studies involving outdoors cultures of different microalgal groups in the Mediterranean basin reported production parameters measured over a short period of time, i.e. days (Krompkamp et al. 2009) or weeks (Rodolfi et al. 2008). There are successful experiences in outdoor conditions of other microalgae classes in lower latitudes, using however a different culture system such as outdoor raceways (Moheimani and Borowitzka 2006). Our study was over a longer period of time compared with other studies at similar

latitudes, from autumn to winter for *H. akashiwo* (approximately 4 months) and during the spring season for *A. minutum* and *K veneficum* (>1 month). The advantage of *H. akashiwo* is that it grows during seasons (autumn–winter) that are usually adverse for other microalgal species due to unfavourable light and temperature conditions. All three species analysed in this study were robust in the system used (bcPBR), with successful outdoor growth probably due to the fact that the strains were isolated from natural populations inhabiting the study area (NW Mediterranean basin). Optimal growth of autochthonous species of seawater microalgae might therefore be achieved under conditions similar to those of their local environment, validating the statement of "build the reactor around the algae" suggested by Morweiser et al. (2010).

The bcPBR used herein allowed good mixing of the cells inside the tubes, with the gas intake in the bottom of the system promoting aeration and dispersion. This type of PBR is known to have a high surface to volume ratio and a small footprint area (Morweiser et al. 2010). In our study, the bcPBR configuration and orientation with respect to the sun avoided overheating inside the tubes at noon, when maximum temperatures were recorded, because the angle of the incident sunlight exposed the tubes to maximum amounts of solar energy mainly in the morning, with a reduction at noon and in the afternoon. In summer, when incident light reaches high radiation values (>4000 $\mu E\ m^{-2}\ s^{-1}$) already before 10:00 AM, the increasing temperature inside the bubble column (41°C mid-July) resulted in photo-inhibition (decolourised cells) and cell death in all strains tested (data not shown). This sequence of events might also be explained by the temperature amplitude, which in summer can be over 25°C during the day. As a result, it is highly advisable to control the amplitude temperature inside the enclosed systems. In order to avoid this amplitude and control the culture temperature, the PBR system implemented in this study was covered with a black Raschel net that diminished the light impacting the surface of the PBR. This reduction in incident light and the consequent temperature control allowed the growth of *A. minutum* and *K. veneficum* during the spring season.

Microalgal growth varies depending on light intensity (light-limited, light-saturated and light-inhibited state) (Soroking and Krauss 1958). Under light-limited conditions, the specific growth rate usually correlates linearly with light intensity. In this study under indoor conditions, all the abiotic parameters were stable. The constant light (low irradiance) and stable temperature maintained the growth rate of all three strains with similar performance. However, while these stable conditions enable predictable production, the amount of energy needed to maintain the system is relatively high (Sevigné et al. 2012). In the present experimental setup, the outdoor conditions resulted in an enhanced growth rate for all three species, due to the higher light intensity and wider spectrum of the natural sun irradiance than the artificial light source used in indoor conditions. The strains were resistant to the variations in temperature and light that occurred outdoors. We suggest a maximum range of temperature variation of 10°C for cultures in bcPBR in outdoor conditions. As regards light radiation, up to 4000 $\mu E\ m^{-2}\ s^{-1}$ of incident light raises the temperature inside the bcPBR and should be considered dangerous for enclosed cultures. This adaptability of the cultures tested permitted a long production period on a year-round basis. However, it would be necessary to avoid the extreme temperature amplitude occurring in the summer season in a bcPBR placed outdoors. For example, the PBR system could be installed in a greenhouse, where abiotic parameters can be controlled or a temperature control device could be installed in order to diminish this amplitude. Nevertheless, this would incur high initial construction costs or high energy consumption. (Molina-Grima et al. 2003; Sevigné et al. 2012).

As regard to the biotic productivity values, interestingly the strains differed in their biomass productivity (in grams per litre per day) depending on the culture conditions. For indoor cultures, productivity was similar for all strains and was higher than reported for other strains of microalgae, such as diatoms (*Chaetoceros* spp., 0.04–0.07 $gL^{-1}\ day^{-1}$), haptophytes (*I. galbana*, 0.14 $gL^{-1}\ day^{-1}$) and xanthophyceae (*Monodus subterraneus*, 0.19 $gL^{-1}\ day^{-1}$) under similar laboratory conditions and despite the inclusion of a CO_2 supply (reviewed by Chen et al. 2011). Higher biomass productivity was obtained under outdoor conditions, with the productivity of *A. minutum* reaching a maximum of 0.35 $gL^{-1}\ day^{-1}$, comparable to the high biomass typically produced by *N. oculata* (Chiu et al. 2009). It is important to note that our culture system has a gas intake that does not require the addition of CO_2 but instead simply uses the CO_2 available in the atmosphere. With the value of CO_2 recorded in our study being slightly higher (420 ppm) than the world average of atmospheric CO_2 (389 ppm), this is probably due that the culture system was located in the city of Barcelona where can be found is an "urban CO_2 island effect" such as found in other populated cities in the world (Balling et al. 2001). We hypothesise that a higher biomass productivity can be obtained for the dinoflagellate and raphidophyte cultures tested if additional CO_2 is provided, increasing carbon availability to the cells and enabling the pH to be adjusted to neutral or even slightly acidic (6.5–7.5 pH). This hypothesis is supported by the pH measurements recorded during the different growth phases of the cultures, indicating a low availability of CO_2 and a consequent basic pH (≈8.39).

The higher biomass productivity obtained in this study was probably due to the high biovolume of the cells cultured in our experimental setup (i.e. *A. minutum* 2,856 μm^3, *H. akashiwo* 1,147 μm^3 and *K. veneficum* 516 μm^3).

Comparing these biovolumes with those of the cells commonly used nowadays for biomass production such as *C. vulgaris* (13 μm^3), *Scenedesmum obliquus* (54 μm^3) (Schmidtke et al. 2010) and *Chlorella minutisima* (1.36 μm^3) and *N. oculata* (13 μm^3) (Lourenço et al. 2002), the three microalgae used in this study are clearly larger in terms of volume. The growth rate of small cells can be high (such as *Nannochloropsis* or *Chlorella*), while other groups which have higher biovolume (such as *Alexandrium* or *Heterosigma*) show a lower growth rate (Tang 1995). But this is not problematic, since when we compare biomass production, expressed in grams of dry weight per litre, by three microalgal strains; these values are quite similar to or even higher than that of the above-mentioned green algae. This is because, although the cell concentration over the same period of time was lower, it was compensated for by a much greater biovolume.

The present data demonstrate the possibility of using marine microalgae with high biomass productivity under outdoor conditions. Thus, the target organisms are not simply those with fast growth, but species that combine high growth under outdoor conditions with high carbon storage (e.g. lipids) due to a high biovolume of the cells, thereby allowing high biomass productivity.

In the present study, the lipid content of the strains tested was >22 % by dry weight, under outdoor and indoor conditions, except for outdoor cultures of *K. veneficum* (Table 1). These data are comparable to those published for other genera such as *Tetraselmis sp.* 14.7 %, *Scenedesmus sp*. 21.1 % and *Nannochloropsis sp*. 21.6 % (Rodolfi et al. 2008). However, the strains of the present study had a higher lipid content than the mean values reported for many other species used for biomass production reviewed by Chen et al. (2011).

Average lipid productivity by our strains was higher except for *K. veneficum*, in outdoor cultures than in indoor cultures. While for outdoor cultures strain-specific differences in lipid productivity were observed, for indoor cultures the differences in lipid productivity between the strains tested were not significant (Table 1). Interestingly, lipid productivity obtained by the dinoflagellates and the raphidophyte studied were higher than that reported for phototrophic marine and freshwater green cells studied by Rodolfi et al. (2008). In their work, the highest lipid productivity reached for a marine strain was *Nannochloropsis sp.* (61 mg L^{-1} day^{-1}), and the highest for a freshwater strain was *Scenedesmus sp.* (53.9 mg L^{-1} day^{-1}). *A. minutum* and *H. akashiwo* cultured under outdoor conditions in this study reached similar or even better results (Table 1) in terms of lipid productivity compared to Rodolfi's work. In fact, in several works, the lipid productivity of the three strains tested in this study is higher than those from what is known as the "green microalgae group" (Illman et al. 2000; Liang et al. 2009; Yoo et al. 2010).

The fatty acid profile of the raw material used for biodiesel production is key in order to obtain the best quality of the obtained biofuel. The percentages of the SAFA, MUFA and PUFA fractions are crucial, and knowing how these fractions change during the cultures of the target strains used for biomass production is recommended (Ramos et al. 2008; Tang et al. 2010). As regards change in lipid composition in microalgae cultures, it is known that the fatty acids profile of eukaryotic microalgae changes during the growth cycle, and the PUFA and SAFA fractions may be present within membrane lipids such as phospholipids, glycolipids or in the cytosol as part of triacylglycerols (Mansour et al. 2003; Guschina and Harwood 2006). The low content of some fatty acids (mainly PUFA) at the end of the growth curve under outdoor conditions (Fig. 4) can be explained by the change observed in the lipid fraction during the growth cycle in eukaryotic cells explained above.

The dominated SAFA fraction in the strains tested, especially C16:0, C18:0 and C14:0, enables us to estimate that the fatty acid profile of the strains is suitable for use as biomass for biodiesel production, particularly in terms of the oxidation stability of the biodiesel obtained (Tang et al. 2010).

Conclusion

We have analysed strains of dinoflagellates and raphidophytes that can be used for biomass production outdoors. These algae are present in seawater all over the world and can be easily isolated. Compared to freshwater algae, they are more competitive in terms of lipid productivity. Moreover, as shown in the present study, they can be cultured in a bubble column photobioreactor system for medium-term biomass production in the Mediterranean area. Under these culture conditions, no drastic changes in terms of growth rate, biomass productivity, lipid content and fatty acid profile were recorded. A long-term study (years) in outdoor condition is desirable to confirm the stability of marine dinoflagellates and raphidophytes in biomass production. Nevertheless, these strains are suitable and competitive candidates for the year-round production of microalgal biomass for third-generation biodiesel fuel or for high-value molecule production.

Acknowledgments We are grateful to the members of the L´Esfera Ambiental Laboratory, Universitat Autònoma de Barcelona, for their help with gas chromatography analyses. We thank S. Fraga for providing the clonal culture AMP4, L.del Río and X. Leal for their help with the experiments and the Experimental Aquarium Area (ZAE) of the ICM-CSIC for the use of their facilities. We gratefully acknowledge the National Scientific and Technical Research Council (CONICYT), Chile, for its support of the scholarship "Beca de Gestión Propia," which finances the PhD studies of C. Fuentes-Grünewald. The work of E. Garcés and S. Rossi is supported by the Ramon y Cajal award from the Spanish Ministry of Science and Innovation.

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