Influence of culture medium recycling on the growth of a marine dinoflagellate microalga and bioactives production in a raceway photobioreactor

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Abstract

Amphidinium carterae is a marine dinoflagellate microalga that produces high-value bioactive polyketides such as amphidinols and amphidinolides, which have remarkable cytotoxic, antifungal and antitumoral activities. This species also produces docosahexaenoic acid and the carotenoid peridinin as high-value byproducts. The development of any sustainable microalgae-based bioprocess must comprise complete valorization of the biomass and reuse of the exhausted culture medium as much as possible to comply with the circular economy concept. In this work we have assessed the effect of recycling the cell-free supernatant on the growth kinetics and production of amphidinols by *A. carterae* photoautotrophically cultured in a pilot-scale raceway-type photobioreactor in semi-continuous mode. Acclimatisation to the recycled medium was first studied on a laboratory scale and was accomplished in the fourth sub-culture, except for pigment content, which decreased throughout the process. No measurably negative effect was detected in the cells. Maximum growth rates and cell productivities increased by 60% with use of 75% spent medium, as a result of mixotrophic consumption of the organic carbon present in that medium, remaining constant thereafter. In the photobioreactor, acclimatisation took 77 days and was also accomplished in the fourth sub-culture, with no negative effects on cell health. Biomass and cell productivities were higher with the recycled medium than with the fresh medium. Pigments decreased throughout the acclimatisation process, probably due to organic carbon-based mixotrophic growth. Amphidinols and docosahexaenoic acid biomass contents were not affected by medium recycling, which will significantly improve the economics of amphidinols production from *A. carterae* cultures on a circular economy basis.

Keywords

Marine dinoflagellate; raceway photobioreactor; *Amphidinium carterae*; medium recycling; Amphidinols; valuable byproducts

1. Introduction

Marine dinoflagellate microalgae have been found to be a source of numerous compounds with fascinating bioactivities [1]. *Amphidinium carterae*, for example, produces an interesting group of polyketide metabolites, namely amphidinolides and amphidinols (henceforth referred to as APDs indistinctly), which exhibit potent anticancer, antifungal and haemolytic activities, amongst others, and have potential use in drug design studies [2]. Our previous findings have demonstrated the possibility of medium- or large-scale production of *A. carterae* using simple and scalable processes [3] with integral use of the biomass [4]. As is the case for traditional microalgae production, the bioprocess should be economically and environmentally optimized using the biorefinery concept and the conclusions of water footprint studies from lifecycle analysis [5]. The dinoflagellate biomass produced contains significant amounts of other high-value compounds with commercial applications [3], such as carotenoid pigments [6] and fatty acids [3,7,8], that could be efficiently recovered to improve the economy of the process [4]. There is also a need to reuse this growth medium to reduce water and nutrient consumption and residue generation [9,10].

Microalgae cultivation is a water-intensive process in which adequate management of water consumption and losses is required to reduce its environmental impact [11]. Since the majority of the water footprint comes from the cultivation system, the maximum amount of spent medium must be recovered and recycled for subsequent reuse. In addition, a large quantity of unused nutrients will be lost if water is not recirculated into the cultivation system after the harvesting process [12]. As such, the reuse of cultivation water reduces the costs and energy associated with pumping input water, adding nutrients, and treating discharged water [13,14]. Although it has to be replenished with consumed nutrients [15–18] in a balanced manner [19], medium

recycling can reduce water consumption sixfold and recover 50-100% of the nutrients [12,20].

It is well known that organic matter (OM) is excreted naturally by microalgae during growth [21] or suddenly released when cell lysis occurs [22]. However, this is strongly dependent on the species [13], culture conditions [23] and growth phase at the time when the biomass is harvested [24,25]. In the exponential growth phase, excretion is active and comprises low molecular weight substances, such as small proteins and peptides [26,27], as a consequence of excess photosynthetic capacity [21,28]. In contrast, in the growth deceleration and stationary phases, high molecular weight species, such as carbohydrates [26,27] and humic substances (HSs), are excreted due to the growth limitation of a nutrient or to decomposition of the accumulated biomass [18,24]. Interestingly, it has been reported that HSs affect dinoflagellate growth [29], with the response depending on the species concerned [30]. Thus, a gradual accumulation of OM [21,31] and counterions [17] occurs in the medium upon recycling, especially in cultures with high biomass concentrations. In general, harvesting algae in the exponential growth phase should produce the most suitable medium for reuse, whereas later phases could more likely cause inhibition [13,32]. Algal growth experiments in spent, or conditioned, media date back to the 1940s [33], with the effects of OM on growth varying greatly depending on the species and culture conditions. Indeed, some authors have found that waste products or toxins may accumulate and affect growth [13,26,34]. When cell growth is inhibited, cells release larger amounts of dissolved organic matter (DOM), which may indeed inhibit cell growth if the medium is recirculated [18,32]. The inhibiting substance depends on the species concerned, but fatty acids, carbohydrates and HSs are the most common [23,26]. *Chlorella vulgaris* [33], *Nannochloropsis* sp. [9,35] and *S. costatum* [36,37] are examples of species that release inhibitory substances. Treatment of the recycled medium with activated carbon,

which may retain inhibiting substances, reduces total organic carbon (TOC) and restores growth [18]. The presence of particulate matter can also reduce production by inducing the formation of cell aggregates, which should therefore be removed [9,38,39]. Furthermore, extracellular products could potentially be a cause and source of bacterial growth, even in controlled cultivation environments such as closed PBR systems [22]. In contrast, recent studies clearly demonstrated the feasibility and benefits of water recycling for the long term reuse of culture media [12,39,40]. Thus, *Tetraselmis* [40] and *Nannochloropsis* [41] could be grown in recycled medium without any harmful effects. Most studies did not measure OM or other compounds left over in the recycled medium, which is essential in order to be able to correlate the concentrations of these compounds with growth response. More research is also needed to determine whether mixotrophic growth may help to achieve higher biomass yields in recycled medium [13]. For example, most marine dinoflagellates are able to grow in mixotrophic environments [42] by consuming DOM [43,44], with mixotrophic growth rates usually being higher than photoautotrophic ones. These microorganisms are also able to store and take up substantial amounts of various sources of N, which is the major growth-limiting nutrient [45,46].

In addition to growth, the recycled medium influences biomass composition and affects the cellular lipid, pigment, carbohydrate and protein contents in microalgae [10,16,18,22,26,39]. However, as far as we are aware, only two laboratory scale studies have been conducted with dinoflagellates. In one of these, the results were inconclusive as no growth was observed after the first reinoculation [30]. In the other study, the addition of HSs to cultures at concentrations of the order of a few milligrams per litre was found to exert a stimulatory effect on growth rates but not on final cell concentrations [25]. In addition, the biochemical composition of the biomass was not analysed. It has recently been demonstrated that valuable products (e.g. APDs, PUFAs

and carotenoids) from *A. carterae* can be robustly produced in conventional pilot-scale raceway photobioreactors (RW-PBR) [3] and efficiently recovered from multi-step processes [4]. Moreover, *A. carterae* has been robustly cultured in a RW-PBR for more than 170 days in semi-continuous mode, obtaining high biomass productivities [3]. This paper reports our evaluation of the influence of medium recycling on the stability, biomass productivity and composition of *A. carterae* cultured in a pilot-scale LED-illuminated RW-PBR in semi-continuous mode.

2. Materials and methods

2.1. Microalgal strain and maintenance

Amphidinium carterae strain from the culture collection belonging to the Plant Biology and Ecology Department of the University of the Basque Country (Dn241EHU) was used. Inocula were grown in standard f/2 medium prepared with Mediterranean seawater, except that the phosphate concentration was increased to 181 µM to achieve an N:P molar ratio of 5 to prevent growth limitation by this nutrient [3].

2.2. Identification of recycling percentage

The feasibility of recycling the supernatant of an RW-PBR for culturing *A. carterae* was initially evaluated on a small scale, performing assays in static T-flasks (ref. 169900 Nunc. EasYFlask 25cm^2 Thermo Fisher Scientific) with a culture volume of 50 mL. Flasks were illuminated laterally using multicolor RGBW-LED strips (red, green, blue and warm white, collectively; Edison Opto Co., Taiwan) arranged horizontally. A 12h/12h light/dark (L/D) cycle was used, with an average irradiance (I_{av}) at the surface of the flasks of 300 µE m^{-2} s^{-1}. The temperature was maintained at 21±1 ºC at the flask illuminated surface. Cell-free supernatant recovered from a RW-PBR after 172 culture days, as reported previously [3], was mixed with seawater in different proportions (25%, 50%, 75% and 100% v/v). Nitrates and phosphates were then measured in each case and replenished as required to achieve the fresh medium

formulation (f/2, N:P = 5). The four resulting medium formulations based on recycled medium (RM) were filtered through a 0.22 µm pore filter (Sartorius Stedim Biotech; model. Sartopore 2 Sterile Midicap) for sterilization and removal of particulate organic matter (POM), then autoclaved. The exhausted nutrients were then replenished with filter-sterilized concentrated stocks. Micronutrients were added in all cases, assuming complete consumption. Fresh medium (f/2, N:P = 5) was used as control. Four sub-cultures of each growth medium were performed to evaluate possible acclimatisation processes. In order to check if the accumulation of DOM was responsible for the culture responses, the same culture medium formulations were prepared but with cell-free supernatant previously passed through a C18 column (Agela Technologies; model. Flash Column 80 g. 40-60µm 60Å) prior to nutrient replenishment.

2.3. Cultivation in the LED-illuminated raceway photobioreactor with recycled medium

The culture experiments performed correspond to the second phase of a long-term (>170 days) culture of *A. carterae* (strain Dn241EHU), as reported recently [3], in which the culture medium was freshly prepared, i.e. non-recycled. The photobioreactor used was a fiberglass paddlewheel-driven raceway PBR (RW-PBR [3]. The PBR had a surface area of 0.44 m^2 and a culture volume of 33 L. A six-bladed paddlewheel with flat blades was operated at a rotation speed of 23.1±0.6 rpm. The system was illuminated using multicolor RGBW-LED strips (Edison Opto Co., Taiwan) horizontally attached to the back of the flat plastic (PVC) PBR cover. After seven sets of experiments, the optimal environmental conditions found were those from SET 7: (i) a sinusoidal diel variation pattern of irradiance imposed with a maximum irradiance of 900 µE $m^{-2}s^{-1}$, at midday, in a 24:0 h L/D cycle, with a daily mean irradiance supplied to the culture medium of 573 µE $m^{-2}s^{-1}$; (ii) f/2×3 (N:P = 5) as culture medium composition; (iii) repeated semi-continuous culture as operation mode.

In the work presented herein, experiments were carried out in the same RW-PBR under the same conditions described above, but using a strategy for recycling the exhausted supernatants obtained after centrifugation in each biomass harvesting. Briefly, experiments started with a 250-day culture broth in the RW-PBR, pure carbon dioxide was injected on demand via a small microporous gas diffuser placed at the bottom immediately behind the paddlewheel to maintain the pH at 8.5. Thermostatted water circulating through a 4.6 m long stainless steel tubular loop (6 mm inner and 8 mm outer diameters) located at the bottom of the channels was used to maintain the culture temperature at 21±1 ºC.

All culture media were prepared using Mediterranean seawater (38 psu) and subsequently autoclaved. The reused medium was prepared following the procedure described in section 2.2. Thus, after harvesting 75% of the whole culture volume, it was centrifuged at 1000×g (RINA model 100 U. 200 SM centrifuge) for cell separation, filtered through a 0.2 µm filter (Sartorius Stedim Biotech; model. Sartopore 2 Sterile Midicap), autoclaved, the exhausted nutrients replenished with filter-sterilized concentrated stock solutions and the resulting mixture returned to the PBR. Harvesting was carried out once the cultures entered a stationary phase. The spent medium was supplemented with phosphate and nitrate stock solutions to achieve the values established (f/2×3, N:P = 5) for the whole culture volume. The remaining nutrients were added in equivalent quantities to those of the medium formulation selected. Four sub-cultures with recycled-replenished medium were carried out to evaluate the acclimatisation and stability of the culture.

Fig. 1 summarizes the strategy followed in the RW-PBR culture. This strategy started with a repeated semi-continuous culture named SET8 (I and II) for 77 days, which was performed as described for SET 7 in the preliminary study (see above) to confirm the robustness of the PBR. The experiments carried out with recycled medium

were named RM1 and RM2 (see Fig. 1). These two sets also comprised two sub-cultures (I and II) each. As can be seen in Fig. 1, the cell-free supernatants were used alternately to allow time for characterization and subsequent preparation for use. In this sense, RM1-I used the cell-free supernatant from SET8-I once it had been replenished with nutrients, and the replenished cell-free supernatant from RM1-I was used in RM2-I. The other sequence of recycled culture medium was from SET8-II to RM1-II and then the replenished cell-free supernatant from RM1-II was used in RM2-II. The average irradiance inside the culture (I_{av}), the effective light attenuation across a cell averaged over the PAR wavelengths (α), and the effective attenuation coefficient of the microalgal suspension averaged over the PAR wavelengths (κ) were calculated as described by Molina-Miras et al. [3].

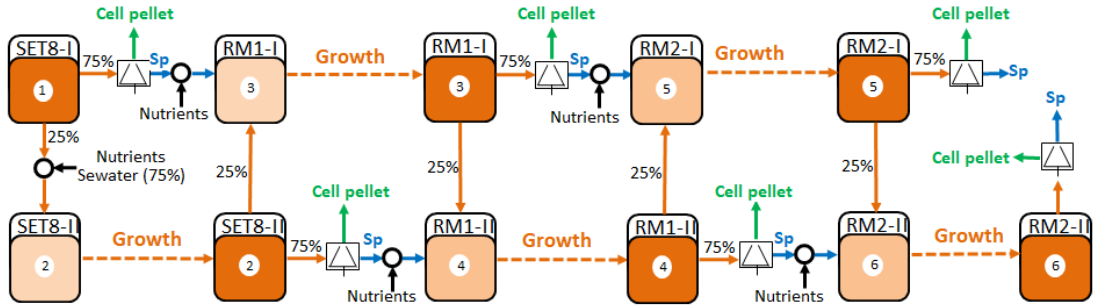


Figure 1. Schematic description of the culture strategy in the raceway photobioreactor for reusing the exhausted culture medium. SET8 is a semicontinuous culture with fresh medium and RM1 and RM2 semicontinuous cultures carried out with recycled medium. I and II refer to two consecutive sub-cultures.

2.4. Hemolytic activity and amphidinol quantification

It has recently been determined that the biomass from *A. carterae* (strain Dn241EHU) contains at least two members of the amphidinol family, namely amphidinol A and its 7-sulfate derivative amphidinol B [47]. Their titers were expressed in terms of hemolytic activity of cell extracts on erythrocytes from defibrinated sheep

blood, as described elsewhere [48]. Briefly, EC50 values for *A. carterae* (i.e. number of cells per well giving 50% hemolysis) and a saponin control were calculated from dose-response Hill curves. Saponin was supplied by Sigma Aldrich (47036, CAS n. 8047-15-2, Saint Louis, MO, USA) and the corresponding EC50 was $8.5 \pm 0.6 \cdot 10^6$ pg per well. An equivalent saponin potency (ESP) expressed in terms of pg saponin per *A. carterae* cell was calculated by dividing the EC50 for saponin by the EC50 for *A. carterae*.

Values for the concentration (% by weight) of APDs in the biomass can be determined from both the ESP values obtained in the hemolytic assays and the absolute value of the integral of the proton peak at δH = 5.07 ppm in the ^{1}H NMR spectrum of aqueous solutions of the NMR standard 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid (TSP, 0.01% w/w) sodium salt. This determination procedure is based on the work of Henderson (2002) [49], since the NMR signal intensity for a defined region of the spectrum represents the total number of the respective nuclei and, thus, the concentration of any substance can be obtained from a known concentration of a standard substance when their spectra are obtained under the same conditions [49]. Therefore, the mass of APDs in the biomass can be obtained from this equation, adapted from Henderson (2002) [49]:

$$APDs, \%\ d.w. = \left(\frac{n_R}{m_b}\right) \times \left(\frac{I_{APDs}}{I_R}\right) \times M_{APDs} \times 100$$

where I_R is the signal intensity of the reference compound, I_{APDs} is the signal intensity of the APDs, n_R is the number of mols of reference standard used in the determinations, M_{APDs} is the average molecular weight of amphidinols A and B ($g \cdot mol^{-1}$) and m_b is the mass of dried biomass in the sample (g). I_{APDs} is the signal intensity of the APDs spectra. This can be obtained from NMR spectra of the cell extracts or from the correlation recently developed by Abreu et al. [47]:

$$I_{APDs} = \frac{ESP - 122.68}{0.0002}$$

This correlation is only valid for this *A. carterae* strain, and eliminates the need to acquire complicated NMR spectra for the biomass extracts.

NMR spectra for the reference standard were recorded using a Bruker Avance III 600 spectrometer operating at a proton frequency of 600 MHz, as described previously [47]. The spectra were automatically phased, baseline-corrected, and calibrated to the TSP signals located at 0.0 ppm for aqueous extracts.This signal strength has been considered to be ideal for the identification and quantification of APDs [47].

2.5. Supernatant characterisation

Prior to proceeding with chemical characterisation of the supernatants, POM was removed by filtration through a 0.2 μm-pore filter (Sartorius Stedim Biotech; model Sartopore 2 Sterile Midicap). The total DOM in the supernatants was quantified using a TOC analyser, as described by González-López et al. [16]. The soluble carbohydrates (SCH) released by cells present in the supernatant were determined using the phenol-sulfuric acid method, with glucose as standard [50]. Proteins (Prot) and humic substances (HSs) were quantified by fluorescence emission measurements in a multiplate reader (BioTek, model SynergyMx). The excitation and emission wavelengths were selected based on the studies of Kong et al. [51] and Chen et al. [52] and corroborated using the excitation-emission matrices (EEMs) of real supernatants. EEMs were performed as described by Henderson et al. [27] using the multiplate reader, with 0.22 μm-filtered seawater as blank. The excitation and emission wavelengths for protein quantification were 280 and 340 nm, respectively. Bovine serum albumin (BSA) (Acros Organics, code 134730100) in the range 0-70 mg/L was used as protein calibration standard. A good correlation was found between emission intensity (*EI*) and protein concentration in the range tested ($C_{\text{Prot}} = 0.0131 \times EI$, mg·L^{-1}; $r^2 = 0.999$). The

excitation and emission wavelengths for humic species were 350 and 440 nm, respectively. Humic acid (Ref 53680, Sigma-Aldrich, Spain) in the concentration range 0-80 mg/L was used for calibration, and a linear relationship was found between EI and humic acid concentration ($C_{HA} = 0.0437 \times EI$, mg·L^{-1}; $r^2 = 0.998$). In both cases, 0.2 μm-filtered seawater was used as blank. Carbohydrate, protein and humic acid measurements were performed in triplicate for every sample.

2.6. Antiproliferative bioassays

Crude methanol extracts obtained from *A. carterae* biomass were used. In the case of supernatants, 24 L of RM2-II supernatant was slowly percolated through prepackaged reverse phase C18 cartridges (Agela Technologies; model. Flash Column 80 g. 40-60μm 60Å), then the salts were removed by washing with distilled water. Any organic compounds adsorbed by the cartridges were eluted with methanol. The solvent was then evaporated *in vacuo*, and the organic extract was freeze-dried to yield 20 mg of dry residue per liter of supernatant. Antiproliferative assays were performed using these crude methanolic extracts, as described elsewhere [53,54]. Four human tumour cell lines obtained from the American Type Culture Collection (ATCC), namely A549 (ATCC CCL-185; lung carcinoma, NSCLC), HT-29 (ATCC HTB-38; colon adenocarcinoma), MDA-MB-231 (ATCC HTB-26; breast adenocarcinoma) and PSN-1 (ATCC CRL-3211; pancreas adenocarcinoma), were used. Cell survival was measured after treatment for 72 h.

2.7. Flow cytometric measurements

Flow cytometry was used to quantify the cell number concentration (N), the average cell equivalent diameter (D_e), the side scatter (SS) related to cell composition and complexity; and the average autofluorescence intensity at specified wavelengths [55]. Five measurements were performed per sample and an average value was used. Cell volume (V_c) was calculated as $\pi D_e^3/6$. All flow cytometric measurements used a

CellLabQuanta SC flow cytometer (Beckman Coulter Inc., Brea, CA, USA) equipped with an argon-ion excitation laser (blue light, 488 nm). The flow rate was kept at a moderate setting (data rate = 600 events s^{-1}) to prevent interference between cells.

Autofluorescence of native pigments and morphology of microalgal cells are good indicators for the acclimatisation of cells to new culture environments [56] as these cell responses are closely related to the pigment content and distribution in cells. As such, cells were excited in the flow cytometer using a 488 nm argon laser, and mean fluorescence intensities were measured in three different wavelength ranges (*FL*1: 525 nm band-pass (BP), *FL*2: 575 nm (BP) and *FL*3: 670 nm long-pass), with each range being characteristic of a group of pigments. The fluorescence detected by *FL*3 and *FL*1-*FL*2 can be used as a proxy for monitoring chlorophyll and carotenoid contents, respectively, when excited at 488 nm [56]. Mathematical relationships between the pigment content in the cell or the effective cell attenuation cross-section and the *FL*1,2,3 and *SS* measurements have been reported recently [55]. For comparison purposes, *FL*1,2,3 intensities were expressed relative to average cell volume (*Vc*).

2.8. Other analytical measurements

The biomass dry weight was determined as described previously [57]. Biomass (d.w.) and cell productivities were calculated in terms of culture volume (i.e. volumetric values) and occupied area (i.e. areal productivities). The ratio between the maximum variable fluorescence (F_V) and maximum fluorescence (F_M) of chlorophyll (i.e. F_V/F_M) in cells was routinely determined as described previously [48]. Phosphate-P and nitrate-N in supernatants were determined as described in a recent study [57]. Measurements were carried out in duplicate, and the average value was used. The NOCHSP elemental composition of the biomass was determined as published previously [57]. Measurements were carried out in triplicate. The saponifiable fatty acids (*FAs*) content and profile were obtained by direct transesterification and gas chromatography (6890N

Series Gas Chromatograph, Agilent Technologies, Santa Clara, CA, USA) as described by Rodríguez-Ruiz et al. [58]. Measurements were carried out in duplicate.

Chlorophylls were quantified spectrophotometrically after extraction with acetone from the cell dry biomass following the method of Hansmann [59], with measurements being carried out in duplicate. Pigments were identified and quantified as described by Molina-Miras et al. [57]. The carotenoid content and profile in cells were determined using a diode array HPLC equipment, following the method described by Zapata et al. [60]. Measurements were carried out in duplicate.

2.9. Kinetic parameters

Culture cell productivities were calculated as:

$$P = \frac{N_f - N_i}{t_f - t_i}$$

Accumulation rates (*AR*) for TOC, HSs and proteins in the cell-free supernatant were calculated as:

$$AR_j = \frac{C_f - C_i}{t_f - t_i} \quad if\ C_f > C_i$$

Consumption rates (*CR*) for the chemical species mentioned above were calculated as:

$$CR_j = -\frac{C_f - C_i}{t_f - t_i} \quad if\ C_f < C_i$$

Specific accumulation rates (*SAR*) were calculated as follows:

$$SAR_j = \frac{C_f - C_i}{(t_f - t_i) \cdot N_{ave}} \quad if\ C_f > C_i$$

And specific consumption rates (*SCR*) as:

$$SCR_j = -\frac{C_f - C_i}{(t_f - t_i) \cdot N_{ave}} \quad if\ C_f < C_i$$

where N is cell concentration, C is the concentration of the given chemical species, t is culture time. Subscripts f and i refer to final and initial time of the time interval for calculation, respectively; subscript j refers to TOC, HSs, Prot or CH; and N_{ave} is the average cell concentration in the time interval.

2.10. Statistical analyses

Multifactor ANOVAs and non-linear regression analyses were performed using Statgraphics Centurion XVI (StatPoint, Herndon, VA, USA).

3. Results

3.1. Optimization of the recycled medium percentage

In this work, the use of recycled medium from the RW-PBR started with a small-scale acclimatisation study. *A. carterae*, maintained over a long period (> 1 year) with f/2 medium, was subjected to four sub-culturing steps using different percentages of recycled medium (%RM). The effect on culture performance and cell health was studied in terms of the kinetics parameters typically used in microalgal culture studies, namely maximum cell concentration attained (N_{max}), F_V/F_M, $FL1$, $FL2$, $FL3$ and SS. The corresponding values are collected in Table 1 for each sub-culture. Cells from the same original inoculum were cultured in T-flasks in batch mode with different %RM in duplicate. To determine whether DOM in the recycled medium had any effect on culture performance, experiments in which the medium was passed through a C18 column prior to culture were also performed (25C18 to 100C18 assays). To analyze the effect of the factors involved (sub-culture and replicate) on the variability of the parameters in each experiment in Table 1, a two-way ANOVA was carried out (values denoted by a different uppercase for each mean value differ significantly; $p<0.05$). No significant differences between the two replicates of each experiment were observed.

N_{max} and the values of fluorescence in the *FL* 1, 2 and 3 channels were affected by RM% and sub-culture (Table 1).Thus, whereas the former increased from RM25 to RM100 and stabilized in the fourth sub-culture, the fluorescence, in general, varied inversely with N_{max}, with significant differences (p<0.05), and did not stabilize, even in the last sub-culture. The final cell concentration in RM100 was 60% higher than the value for the control culture, whilst the *FL*2 value (the most affected by %RM) for that %RM was half the CTRL one. In contrast, when the recycled medium at any percentage was passed through the C18 column, N_{max} and *FL*1,2,3 were less affected and found to stabilize by the third sub-culture. The maximum cell concentration for 100C18 was 32% higher than that for the control. With average values for all medium compositions and sub-cultures of 0.59 ± 0.02 (n = 72) for F_V/F_M and 4.12± 0.32 (n = 72) for *SS*, these variables were not affected by the recycled medium.

Table 1. Trend in the acclimatisation of *Amphidinium carterae* to different recycled medium percentages (RM%) through four sub-cultures (1, 2, 3 and 4). Control experiments (CTRL) were carried out with fresh f/2 medium (N:P = 5). Experiments with recycled medium passed through a C18 column prior to culture are named as %C18. The kinetics parameters were measured in broth samples withdrawn at the end of every sub-culture. Data points are averages of duplicate cultures, with errors corresponding to their standard deviation. Values denoted by a different uppercase letter at each point, for the same parameter, differ significantly (p < 0.05) in the two-way ANOVA. N_{max}: maximum cell concentration; F_V/F_M: maximum photochemical yield of photosystem II; *FL*1,2,3: cell fluorescence intensities measured in the *FL*1, *FL*2 and *FL*3 channels of the flow cytometer normalized to the cell average volume; *SS*: side-scatter of the cells. Shaded values indicate that the variable has stabilized, therefore there are no significant differences with respect to previous and subsequent sub-cultures.

	Sub-culture			
N_{max} (×10^5), cell·mL^{-1}	1	2	3	4
CTRL	2.97±0.16 ^{a}	2.32±0.07 ^{b}	2.47±0.08 ^{b}	2.76±0.15 ^{a,b}
RM25	2.45±0.05 ^{a}	2.04±0.12 ^{b}	2.13±0.08 ^{b}	2.57±0.06 ^{a}
RM50	2.70±0.01 ^{b}	2.76±0.06 ^{b}	2.27±0.12 ^{c}	2.94±0.01 ^{a}
RM75	3.57±0.03 ^{b}	3.46±0.04 ^{b}	3.52±0.08 ^{b}	3.91±0.17 ^{a}
RM100	3.82±0.04 ^{b}	3.32±0.08 ^{c}	4.33±0.02 ^{a}	4.49±0.08 ^{a}
18C25	2.94±0.11 ^{a}	2.96±0.02 ^{a}	2.57±0.08 ^{b}	2.81±0.05 ^{a,b}
18C50	3.53±0.05 ^{a}	3.30±0.02 ^{b}	3.19±0.05 ^{b}	3.21±0.07 ^{b}
18C75	3.45±0.07 ^{a}	3.18±0.02 ^{b}	3.43±0.01 ^{a}	3.43±0.06 ^{a}
18C100	3.41±0.02 ^{b}	3.65±0.10 ^{a}	3.65±0.02 ^{a}	3.54±0.02 ^{a}

	Sub-culture			
F_V/F_M	1	2	3	4
CTRL	0.59±0.02 [a]	0.59±0.01 [a]	0.61±0.01 [a]	0.60±0.01 [a]
RM25	0.58±0.02 [a]	0.54±0.01 [a]	0.60±0.02 [a]	0.60±0.01 [a]
RM50	0.57±0.01 [a]	0.56±0.01 [b]	0.59±0.01 [a]	0.59±0.01 [a]
RM75	0.56±0.01 [b,c]	0.55±0.01 [c]	0.60±0.02 [a,b]	0.60±0.01 [a]
RM100	0.56±0.01 [b]	0.54±0.01 [c]	0.58±0.01 [a]	0.59±0.01 [a]
18C25	0.58±0.01 [b]	0.58±0.01 [b]	0.64±0.01 [a]	0.63±0.01 [a]
18C50	0.60±0.01 [b]	0.59±0.01 [b]	0.60±0.01 [a]	0.59±0.01 [a]
18C75	0.58±0.01 [a]	0.59±0.03 [a]	0.62±0.03 [a]	0.61±0.01 [a]
18C100	0.58±0.01 [a]	0.58±0.01 [a]	0.61±0.02 [a]	0.60±0.01 [a]
	Sub-culture			
FL1 (x10^{-3}), µm^{-3}	1	2	3	4
CTRL	12.95±3.34 [a]	12.60±1.11 [a]	15.02±2.46 [a]	13.87±1.49 [a]
RM25	12.61±0.86 [b]	12.69±0.56 [a]	12.97±0.62 [a]	12.27±0.01 [a]
RM50	17.32±0.54 [a]	16.03±1.04 [a,b]	14.71±1.70 [b]	10.74±0.96 [c]
RM75	19.55±0.13 [a]	15.04±0.18 [b]	14.11±0.11 [b]	9.99±0.64 [c]
RM100	15.03±2.19 [a]	9.95±1.02 [b]	12.00±0.27 [a,b]	9.30±1.04 [b]
18C25	10.62±0.42 [a]	10.39±0.22 [a]	10.57±1.33 [a]	10.75±0.35 [a]
18C50	12.49±1.87 [a]	12.11±0.25 [a]	14.59±2.63 [a]	13.89±1.91 [a]
18C75	13.51±0.43 [a]	12.26±0.21 [b]	12.74±0.01 [b]	12.78±0.53 [a,b]
18C100	14.06±3.24 [a]	17.04±0.43 [a]	19.63±2.01 [a]	14.85±2.91 [a]
	Sub-culture			
FL2 (x10^{-3}), µm^{-3}	1	2	3	4
CTRL	4.41±1.00 [a]	4.22±0.26 [a]	4.08±0.77 [a]	6.36±0.06 [a]
RM25	4.30±0.44 [b]	4.43±0.35 [b]	5.03±0.20 [a,b]	5.44±0.04 [a]
RM50	6.32±0.11 [a]	6.73±0.40 [a]	6.52±0.95 [a]	4.85±0.80 [b]
RM75	8.33±0.39 [a]	7.52±0.28 [b]	5.99±0.15 [c]	4.64±0.71 [d]
RM100	6.39±1.37 [a]	3.95±0.69 [b]	4.10±0.00 [a,b]	3.10±0.26 [b]
18C25	3.09±0.08 [a]	3.13±0.01 [a]	3.24±0.46 [a]	3.56±0.04 [a]
18C50	3.80±0.40 [a]	3.76±0.12 [a]	4.49±0.66 [a]	4.53±0.66 [a]
18C75	4.30±0.02 [a]	3.93±0.06 [b]	4.21±0.13 [a]	4.24±0.10 [a]
18C100	4.63±1.03 [a]	5.48±0.20 [a]	6.49±0.62 [a]	4.93±1.01 [a]
	Sub-culture			
FL3 (x10^{-3}), µm^{-3}	1	2	3	4
CTRL	20.80±5.50 [a]	16.07±0.61 [a]	22.36±4.21 [a]	21.30±1.73 [a]
RM25	18.07±0.86 [a]	16.81±0.50 [a]	17.28±0.67 [a]	16.33±0.14 [a]
RM50	25.45±0.01 [a]	21.02±1.63 [b]	18.18±1.33 [c]	15.30±0.40 [d]
RM75	25.66±0.45 [a]	17.44±0.65 [b]	19.62±1.14 [c]	14.64±0.14 [d]
RM100	19.12±0.65 [a]	14.48±0.06 [b]	18.96±1.21 [a]	16.68±1.50 [a,b]
18C25	17.59±0.74 [a]	15.61±0.09 [b]	13.55±1.39 [c]	14.91±0.52 [b,c]
18C50	22.48±3.69 [a]	18.21±0.02 [a]	19.96±2.85 [a]	20.63±2.62 [a]
18C75	24.32±1.97 [a]	18.14±0.07 [b]	19.06±0.55 [b]	19.69±1.16 [b]
18C100	26.36±6.77 [a]	24.49±1.11 [a]	26.44±1.37 [a]	21.45±3.91 [a]
	Sub-culture			
SS	1	2	3	4
CTRL	4.23±0.05 [a]	4.18±0.19 [a]	4.07±0.00 [a]	4.03±0.07 [a]
RM25	4.37±0.01 [c]	4.46±0.02 [b]	4.53±0.04 [a]	4.53±0.01 [a,b]
RM50	4.31±0.04 [d]	4.55±0.01 [b]	4.64±0.05 [a]	4.42±0.04 [c]
RM75	4.23±0.05 [a]	4.54±0.02 [a]	4.13±0.68 [a]	4.32±0.00 [a]
RM100	4.31±0.02 [a,b]	4.34±0.11 [a]	4.22±0.08 [a,b]	4.02±0.12 [b]
18C25	3.94±0.03 [b]	4.16±0.01 [a]	4.20±0.00 [a]	4.22±0.04 [a]
18C50	3.83±0.01 [a]	3.97±0.02 [a]	3.92±0.07 [a]	3.87±0.06 [a]
18C75	3.81±0.05 [a]	3.94±0.02 [b]	3.78±0.04 [a]	3.83±0.01 [a]
18C100	3.70±0.05 [a]	3.92±0.04 [a]	3.71±0.06 [a]	3.73±0.10 [a]

Maximum growth rates (μ_{max}), calculated in the exponential growth phase, and cell productivities (P_{cell}), calculated from N at the culture onset and N_{max}, were obtained from the time-evolution of the cell concentrations corresponding to the four sub-cultures for all medium compositions. Figure 2 shows these two parameters relative to the control culture. Relative P_{cell} shows a slight increase with RM25 and RM50 compared to the control with fresh medium, although there are no statistically significant differences between these two values. The use of 75% recycled medium (RM75) increased P_{cell} roughly two- to threefold compared with the control culture, the value for which was $3.63 \pm 0.36 \cdot 10^4$ cell·mL^{-1}·day^{-1}. The culture in 100% recycled medium (RM100) did not improve P_{cell} significantly compared to RM75. The cultures performed with the C18 treatment are also shown in Fig. 2. Thus, 25% recycled medium (18C25) had no significant effect on culture performance compared to the CTRL. Further increases in the percentage of spent medium slightly improved P_{cell} to a similar degree as for the RM25 culture, with no differences between them. Apparently, the C18 column retained some of the growth-stimulating substances. μ_{max} for the control culture was 0.53 ± 0.04 day^{-1}. The relative values for the other medium formulations followed the same trend as P_{cell}, except for 100% RM (which presented a lower value than RM75), 25C18 (which presented a higher value than the control) and 100C18 (which presented a similar value to RM100). The highest value (170% that of the control) was obtained for RM75.

As centrifugation takes a short time on a laboratory scale, sub-cultures can be performed by sterile centrifugation of the cell broth and subsequently resuspending the pellet in the selected culture medium in another flask. On larger scales, however, centrifugation takes longer, there is no replacement for the photobioreactor and thus the risk of contamination is high. On these scales, it is easier to carry out sub-cultures by removing a percentage of the culture broth and adding the cell-free supernatant

replenished with nutrients afterwards. As such, 75% of the culture broth was removed from the RW-PBR and replaced with nutrient-supplemented used supernatant. Thus, as 25% of spent medium remains in the RW-PBR, 100% recycled medium was used for the culture experiments on a larger scale in the RW-PBR as this resulted in the highest P_{cell} and μ_{max}.

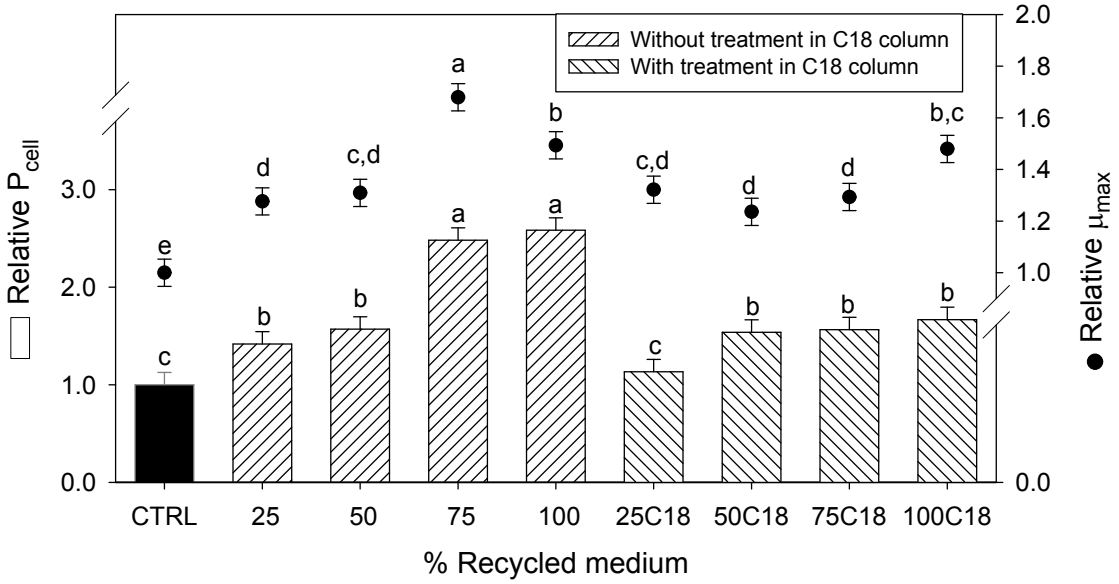


Figure 2. Cell productivities (P_{cell}) and maximum growth rates (μ_{max}) relative to the control culture as a function of the percentage of recycled medium with or without prior treatment on a C18 column. Data points are the averages of four sub-cultures for each percentage, and intervals represent 95% confidence based on Fisher's least significant difference (LSD) procedure. Values denoted by a different letter differ significantly. Acronyms correspond to control experiments (CTRL) carried out with fresh f/2 medium (N:P = 5), experiments with different recycled medium percentages (RM%) and experiments with recycled medium passed through a C18 column prior to culture (%C18).

3.2. Culture in the RW-PBR

Culture results from the LED-illuminated RW-PBR are displayed in Fig. 3, which uses the nomenclature of each sub-culture according to Fig. 1. An average N_{max} of around $4.7\pm0.2\cdot10^6$ cell·mL^{-1} was attained for the three sets, although the maximum N_{max} ($5\cdot10^6$ cell·mL^{-1}) was found for SET8-I using fresh medium, this value being similar to that reported for a similar experiment (SET 7) [3]. However, the average N_{max} values in RM1 and RM2 were reached more quickly and, as a result, volumetric and

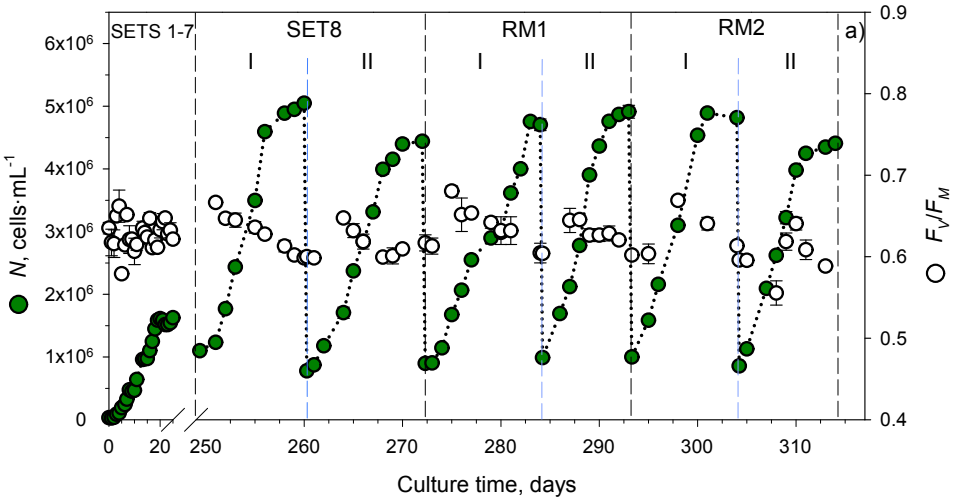
areal cell productivities (P_{cell}) were 13% and 11% higher, respectively, than for SET8 (see Table 2), although there were no statistically significant differences between RM1 and RM2. In contrast, the corresponding biomass productivities (P_b) increased from SET8 to RM2 ($p<0.05$), with the average maximum P_b (0.039 $g \cdot L^{-1} \cdot day^{-1}$) in RM2 being 22% higher than the value for SET8. This was caused by a slightly larger cell size in RM2 ($7.84 \pm 0.33\ 10^2\ \mu m^3$) than in SET8 ($7.04 \pm 0.21 \cdot 10^2\ \mu m^3$) (Table 2).

F_v/F_m was highest at the onset of sub-culture, subsequently decreasing with increasing culture time in a similar manner for all three culture sets. The value ranged between 0.56 and 0.69, with an average value of 0.614±0.030 for the three sets (Fig. 3a). The evolution of I_{av} over culture time was very similar in the three sets: peaking at the onset of every sub-culture when N was lowest and decreasing sharply to a similar minimum value (always higher than 100 $\mu E \cdot m^{-2} \cdot s^{-1}$) in the stationary phase due to the increased mutual shading between cells (see Fig. 3b). During the growth deceleration phase, I_{av} increased slightly even though N continued to increase as κ decreased over time. As can be seen from Fig. 3b, α evolves in parallel with I_{av}, decreasing a few days after the beginning of each batch to reach a minimum of around $7 \cdot 10^{-12}$ $m^2 \cdot cell^{-1}$ in the stationary growth phase. This minimum value was consistent between sets, whereas the maximum values showed more variability. The κ value, which directly depends on α and cell concentration, increased to a maximum in the growth deceleration phase and decreased at the end of the culture (Fig. 3b). The patterns described above were in line with those reported previously for this kind of culture [3].

As can be seen from Fig. 3c, nitrates and phosphates were replenished in the recycled medium prior to every sub-culture to the concentrations corresponding to the formulation used in SET8 (2646 µM NO_3^- and 549 µM PO_4^{3-}). The evolution of these substances was parallel and almost coincident in the three sets, being rapidly taken up by the cells in the first three days of every sub-culture. Nitrates were completely

depleted by the end of the culture, thus indicating a mainly N-limited growth. Nitrate in SET8 and RM2-II was completely consumed at the onset of the linear growth phase. Close to 5% of the initial phosphate remained in the medium, although for RM1-I it was exhausted. Initial phosphate (R_{Po}) and nitrate (R_{No}) uptake rates in the exponential growth phase, averaged over all sets, were 179±54 and 877±244 µmol L^{-1}·day^{-1}, respectively. However, phosphate was consumed more rapidly in RM1 and RM2 than in SET8, probably due to a faster growth in the latter two sets. As such, the maximum R_{Po} and R_{No} values (550 µmol P·L^{-1}·day^{-1} and 2200 µmol N·L^{-1} day^{-1}, respectively) were found in RM2-II. The corresponding specific initial nutrient uptake rates (r_{Po} and r_{No}) followed the same trend, with r_{Po} and r_{No} values averaged over all sets at around 0.21 and 0.94 pmol $cell^{-1}$·day^{-1}, respectively. Nitrate biomass yields ($Y_{b/N}$) were statistically identical for all three sets. In contrast, $Y_{b/P}$ differed between RM1 and RM2 (p<0.05) as phosphate was depleted in RM2-I (see Table 2).

The cell volume was not greatly affected upon reusing the medium, although it was statistically significantly different in RM2 compared to the other sets (see Table 2), thus implying differences in biomass and cell productivities (see Table 2).



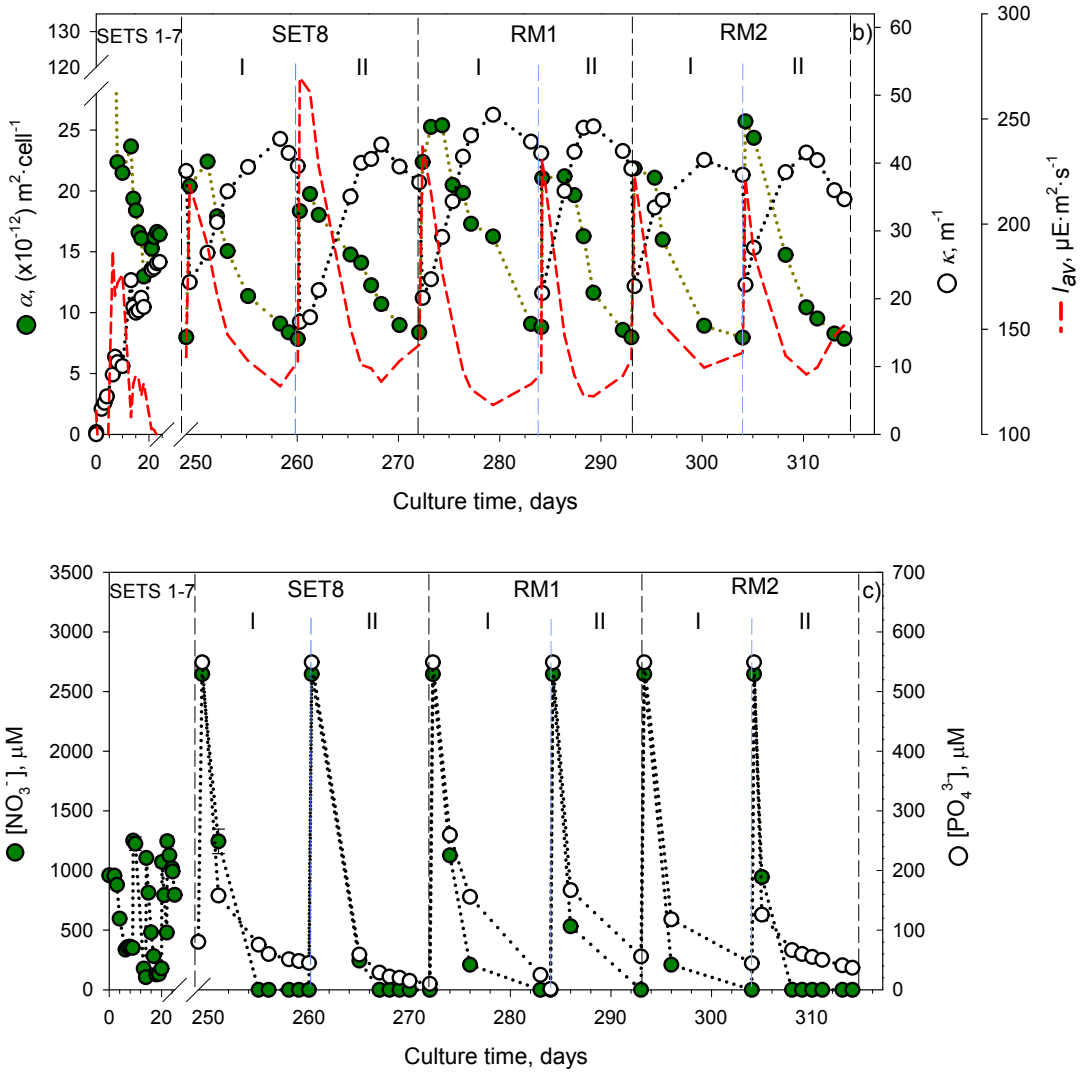


Figure 3. Dynamics for sequential culture of the microalga *Amphidinium carterae* in a pilot-scale LED-illuminated raceway photobioreactor. Temporal changes in (a) cell concentration (N), maximum photochemical yield of photosystem II (F_V/F_M); (b) average irradiance available for the cells (I_{av}), effective light attenuation across a cell (α) and effective attenuation coefficient of the microalgal suspension (κ); (c) dissolved nitrate ($[NO_3^-]$) and phosphate ($[PO_4^{3-}]$) concentrations in the supernatant. The vertical dotted lines delimit the different experimental sets performed. SETs 1-7 correspond to previously published culture conditions and strategies [3]. The last two sub-cultures of SET8 (carried out with fresh medium) are represented, with SETS RM1 and RM2 corresponding to two consecutive semi-continuous cultures with recycled medium. I and II refer to two consecutive sub-cultures. Data points are averages, and vertical bars are standard deviations (SD) for duplicate samples.

Table 2. Culture productivities, average cell diameters and biomass yields of nitrate and phosphate calculated at the onset of the stationary growth phases. Values are averages with standard deviations for duplicate cultures. Values denoted by a different uppercase letter for each value, for a same kinetic parameter, differ significantly (p<0.05) in the two-way ANOVA. P_{cell}, cell productivities; P_b, biomass productivities; μ_{max}, maximum specific growth rates; V_C, average cell volumes; $Y_{b/N}$, biomass nitrogen yields and $Y_{b/P}$ biomass phosphate yields. SET8 is a semi-continuous culture with fresh medium and RM1 and RM2 semi-continuous cultures carried out with recycled medium.

SET	P_{cell} ($\times 10^4$), cell·mL^{-1}·d^{-1}	P_b, g·L^{-1}·day^{-1}	P_{cell} ($\times 10^6$), cell·m^{-2}·day^{-1}	P_b, g·m^{-2}·day^{-1}	μ_{max}, day^{-1}	V_C (x10^2), µm^3	$Y_{b/N}$	$Y_{b/P}$
8	25.67±0.65 ^{b}	0.032±0.001 ^{c}	19.25±0.49 ^{b}	2.42±0.06 ^{c}	0.28±0.02 ^{b}	7.04±0.21 ^{b}	2.86±0.15 ^{a}	9.78±0.09 ^{a,b}
RM1	28.96±0.36 ^{a}	0.036±0.001 ^{b}	21.72±0.27 ^{a}	2.66±0.03 ^{b}	0.32±0.03 ^{a,b}	7.07±0.03 ^{b}	2.82±0.15 ^{a}	9.44±0.12 ^{b}
RM2	28.17±0.34 ^{a}	0.039±0.001 ^{a}	21.12±0.26 ^{a}	2.90±0.03 ^{a}	0.34±0.01 ^{a}	7.84±0.33 ^{a}	3.11±0.21 ^{a}	10.70±0.29 ^{a}

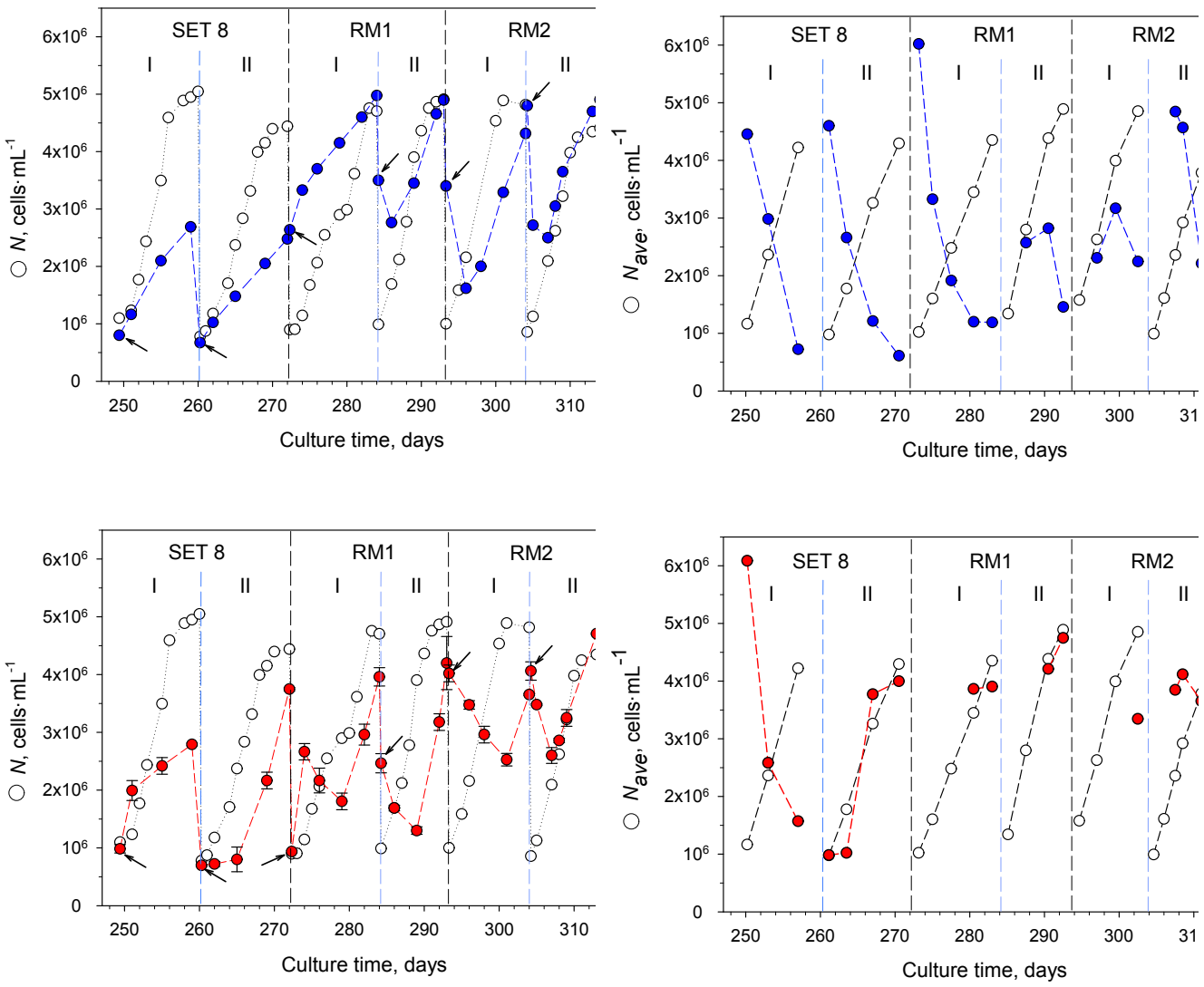
3.3. Cell-free supernatant characterization

Fig. 4a shows the accumulation of TOC in all sub-cultures. In SET8, TOC varied similarly for the two cultures carried out with fresh medium (SET8-I,II), increasing from a minimum value of nearly 18 $mg \cdot L^{-1}$ at the onset to a maximum of around 51 $mg \cdot L^{-1}$ at the end (Fig. 3a). In RM1, TOC also accumulated from the start to the end of the sub-culture, although it reached a much higher final concentration (close to 99.5 $mg \cdot L^{-1}$). As RM1 was set up using the cell-free supernatant from SET8-I (see Fig. 1), the starting TOC concentration is virtually the same as the final TOC concentration in SET8-I. In the next three culture experiments, TOC was consumed during the first 3-4 days of culture and then increased practically reaching the same maximum value ($\geq$ 90 $mg \cdot L^{-1}$). Accumulation rates, AR, for TOC averaged 3.5 $mg \cdot L^{-1} \cdot day^{-1}$ in SET8, progressively increasing through RM1 to an average of 7.5 $mg \cdot L^{-1} \cdot day^{-1}$ in RM2. *SAR*s also varied for the various sub-cultures (see Fig. 4b). In general, the values decrease in a very similar fashion over culture time, with the exception that they are highest at the culture onset in SET8-I,II and RM1-I, when the maximum SAR_{TOC} value of 8 $ng\ cell^{-1} \cdot day^{-1}$ was obtained. In RM1-II and RM2-I, SAR_{TOC} increased slightly and then decreased until harvesting time. In contrast, SAR_{TOC} in RM2-II decreased continuously from the start of the deceleration phase to harvesting time, and TOC was consumed at an average CR of 8.3, 6.0 and 16.6 $mg \cdot L^{-1} \cdot day^{-1}$ during RM1-II and RM2-I,II, respectively. The maximum SCR_{TOC} of 54 $ng \cdot cell^{-1} \cdot day^{-1}$ corresponds to RM2-II.

A comparison between Figures 4a and 4c shows that the temporal evolutions of HSs and TOC concentrations are almost parallel, except for RM1-I. The concentrations of these species in RM1 and RM2 decreased during the first 3-4 days and then increased, reaching a maximum in the stationary growth phase. The maximum and minimum HSs values fluctuated more than for TOC. Thus, the maximum AR_{HS} of 10 $mg \cdot L^{-1} \cdot day^{-1}$ corresponded to RM1-II, with this value being very similar to those for the

two subcultures of RM1-I and RM2 (~5 mg·L^{-1}·day^{-1}). The HSs values in SET8 were lower (~3 mg·L^{-1}·day^{-1}). The SAR_{HS} values in RM1 and RM2 were similar (1.68±0.5 ng·cell^{-1}·day^{-1}), whereas for SET8 this parameter differed between batches, decreasing over time in SET8-I and increasing in SET8-II. Consumption rates were similar in the first three cultures carried out with recycled medium (~2.7 mg·L^{-1}·day^{-1}) and higher in RM2-II (7.3 mg·L^{-1}·day^{-1}). SCRs decreased over time in RM1 and RM2, with a maximum of 10.5 ng·cell^{-1}·day^{-1}.

Proteins and carbohydrates accumulate to a lesser extent, especially the latter, the concentration of which is always below 1 mg·L^{-1}. Protein concentrations followed an inverse trend to nitrates, TOC and HSs in the medium, accumulating markedly in the first days of culture. However, there were some differences between sets. Thus, whereas the concentrations of these species increased throughout the whole culture time in the two sub-cultures for SET8, they only accumulated during the first few days in the remaining sub-cultures, subsequently being consumed until the stationary growth phase (Fig. 4e). There appeared to be an adaptation effect in protein accumulation, as the maximum concentration decreased from the first to the third sub-culture carried out with recycled medium. The highest value corresponded to RM2-II (approx. 6 mg·L^{-1}). AR_{Prot} ranged between 0.06 for the first sub-culture of SET8 and 1.28 mg·L^{-1}·day^{-1} for the second sub-culture of RM2 (see Fig. 4f), in which nitrates were completely depleted (Fig. 3c). *SAR* decreased steadily in all experiments except for RM1-I, that is the first subculture in which recycled medium was used (Fig. 4f). *SAR*s ranged between 1.34 for the first sub-culture of SET8 and 2.57 ng·cell^{-1}·day^{-1} for RM2-II (Fig. 4f).



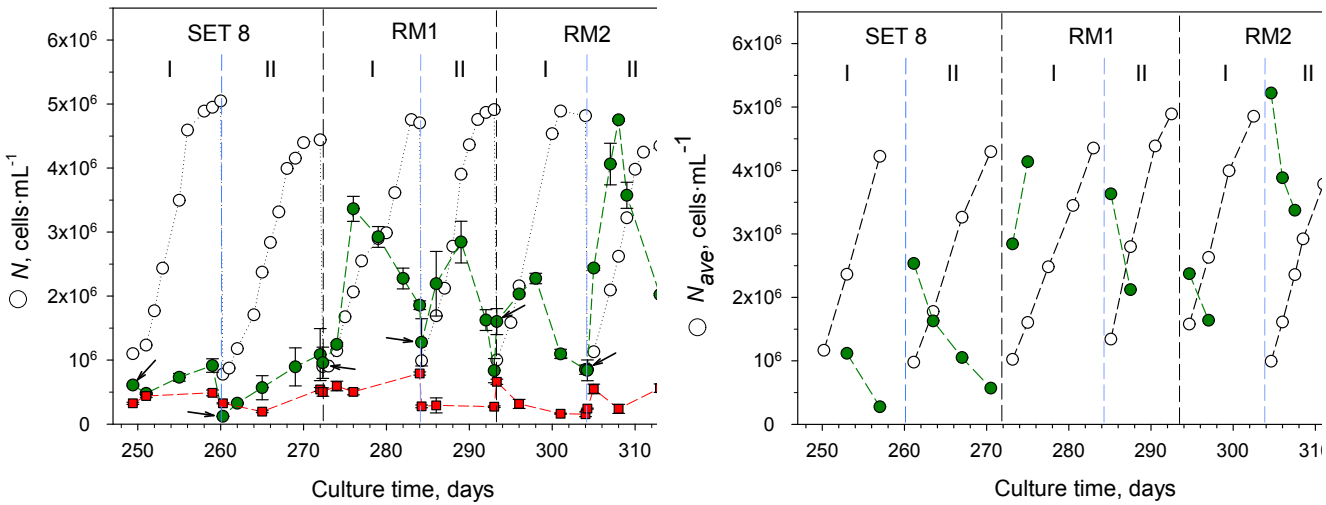


Figure 4. Evolution with culture time of: a) Total Organic Carbon (TOC); b) specific accumulation rate of TOC in the culture medium (SAR_{TOC}); c) concentration of humic substances; d) specific accumulation rate of HSs in the culture medium (SAR_{HS}); e) protein and carbohydrate concentration; and e) specific accumulation rate of proteins in the culture medium (SAR_{Prot}). SET8 was carried out with fresh medium and sets RM1 and RM2 correspond to two consecutive semi-continuous cultures carried out with recycled medium. I and II refer to two consecutive sub-cultures within each set. Error bars correspond to standard deviation of two measurements. Cell concentrations have also been included for clarification purposes. Arrows indicate the substance concentration after dilution with the recycled medium.

3.3. Biomass characterization

3.3.1. Elemental analysis

Table 3 reports the elemental composition of the *A. carterae* biomass harvested from the six sub-cultures with averaged data for each condition. As can be seen, the use of recycled medium does not affect the biomass elemental mass composition to a statistically significant degree. Only N is continuously reduced in the biomass from SET8 to RM2 (~15% reduction). When molar ratios are calculated, C is increased compared to P and N. Thus, there are statistically significant differences in the C:P and C:N molar ratios for the biomass harvested in RM2 compared with the other two sets. Apparently, once the cells have acclimatised to growth in a supernatant with high TOC, they increase their C content at the expense of reducing N and P. This is also evident when the biomass P-molar formulas are calculated, with C (32.9 to 38.3), H (60.7 to 70.8) and O (16.7 to 18.3) increasing from SET8 to RM2.

Table 3. Elemental composition, elemental molar ratios and P-molar formula of harvested *A. carterae* biomass. Values are the average for the two sub-cultures of each set. The error is the SD of three determinations per sub-culture. Values for each element percentage with different uppercase letters differ significantly. SET8 was carried out with fresh medium and sets RM1 and RM2 correspond to two consecutive semi-continuous cultures carried out with recycled medium.

Elements	Experimental SET		
	8	RM1	RM2
% N	5.5±0.2 ^{a}	5.2±0.1 ^{b}	4.8±0.2 ^{c}
% C	48.7±0.5 ^{a}	49.3±1.3 ^{a}	50.2±0.1 ^{a}
% H	7.5±0.0 ^{a}	7.6±0.1 ^{a}	7.7±0.0 ^{a}
% S	1.0±0.2 ^{a}	1.3±0.1 ^{a}	1.3±0.0 ^{a}
% P	3.8±0.1 ^{a}	3.6±0.3 ^{a}	3.4±0.0 ^{a}
C:P	32.9±0.9 ^{b}	35.2±1.6 ^{b}	38.3±0.4 ^{a}
C:N	10.3±0.5 ^{c}	11.0±0.6 ^{b}	12.2±0.5 ^{a}
N:P	3.2±0.2 ^{a}	3.2±0.3 ^{a}	3.2±0.2 ^{a}
P-molar formula	$\mathbf{C_{32.9}O_{16.7}H_{60.7}N_{3.2}S_{0.3}P_1}$	$\mathbf{C_{35.2}O_{17.5}H_{64.8}N_{3.2}S_{0.4}P_1}$	$\mathbf{C_{38.3}O_{18.3}H_{70.8}N_{3.2}S_{0.4}P_1}$

3.3.2. Fatty acids

Fig. 5a shows the averaged saponifiable fatty acids (FAs) contents determined for the *A. carterae* biomass corresponding to culture SETS 8 to RM2. FAs were grouped into three classes, namely saturated (SFAs), monounsaturated (MUFAs) and polyunsaturated (PUFAs) fatty acids (Fig. 5a inset). A comparison of the distribution of FA classes between sets showed no statistically important differences. Thus, PUFAs showed a slight decrease and SFAs a slight increase, whereas MUFAs were not affected when the recycled medium was used (i.e. from SET8 to RM2). PUFAs were the most important group, representing more than the 50% of all FAs, followed by SFAs, with more than the 30% of the total; MUFAs always represented less than 20% of all FAs (Fig. 5a inset).

Irrespective of the set considered, the majority SFA was 16:0 (PA) (Fig. 5a). The dominant MUFA was 18:1*n*9, although it represented only about 1% of the biomass d.w. and, as such, was included in the group of minority FAs. The biomass was always rich (in this order) in 22:6*n*3 (DHA), 18:4*n*3 (SDA) and 20:5*n*3 (EPA) (Fig. 5a). Minority FAs (14:0, 18:0, 18:1*n*9, 20:1*n*9 and others) represented less than 3.5% of the biomass d.w. With an average content of ~5% d.w., there were no statistically significant differences between the DHA content in the biomass between sets (Fig. 5a), with productivity being highest in RM2 (1.85 $mg \cdot L^{-1} \cdot day^{-1}$). With regard to PA, the next FA in abundance, statistically significant differences were found between sets. Thus, the abundance of this FA was highest in RM2 biomass (~4% d.w.), followed by SET8 and RM1. SDA decreased linearly, with statistically significant differences being found between SET8 (2.6% d.w.) and RM2 (1.75% d.w.). The average EPA content (~1.8% d.w.) did not differ significantly between the three sets. The minority FA content only differed significantly between SET8 (3.5% d.w.) and RM1 (3.1% d.w.).

The total FA content (FA_T) did not differ significantly between sets, with an average content of 15.9±0.8% d.w. for all sets (Fig. 5d).

3.3.3. Pigments

Fig. 5b displays the cell pigment percentage by biomass dry weight corresponding to the three majority pigments in the cultures for experimental sets 8 to RM2 (the data represented in Fig. 5b were calculated as the average of the two semi-continuous cultures for each set). Chlorophyll a (Chl-a) was the main pigment, followed by Peridinin (Per) and Chlorophyll c2 (Chl-c2), with all samples analyzed also containing diadinochrome, β-carotene, peridininol, diatoxanthin, dinoxanthin, pyrrhoxanthin and diadinoxanthin, with these representing less than 0.7% d.w. (minority pigments in Fig. 5b). There were significant differences in the Chl-a content between sets, decreasing linearly from 1.5% d.w. in SET8 to 0.65 d.w. in RM2 (Fig. 5b). The evolution of Per mirrored that of Chl-a, with a maximum content of 0.95% d.w. (5.5 $mg \cdot L^{-1}$) in SET8 and a minimum of 0.4% in RM2-II. Chl-c2, the least abundant of the majority pigments, also decreased from RM1-I (0.6% d.w.) to RM2-II (0.12% d.w.) in a statistically significant manner. The minority pigments followed the same trend as Chl-a.

The total pigment yields, expressed as percentage of biomass dry weight, differed significantly between the three sets, decreasing almost linearly from a value of 3.5% d.w. in RM1-I to 1.2% d.w. in RM2-II, mainly because Chl-a and Per, the main pigments, followed this trend.

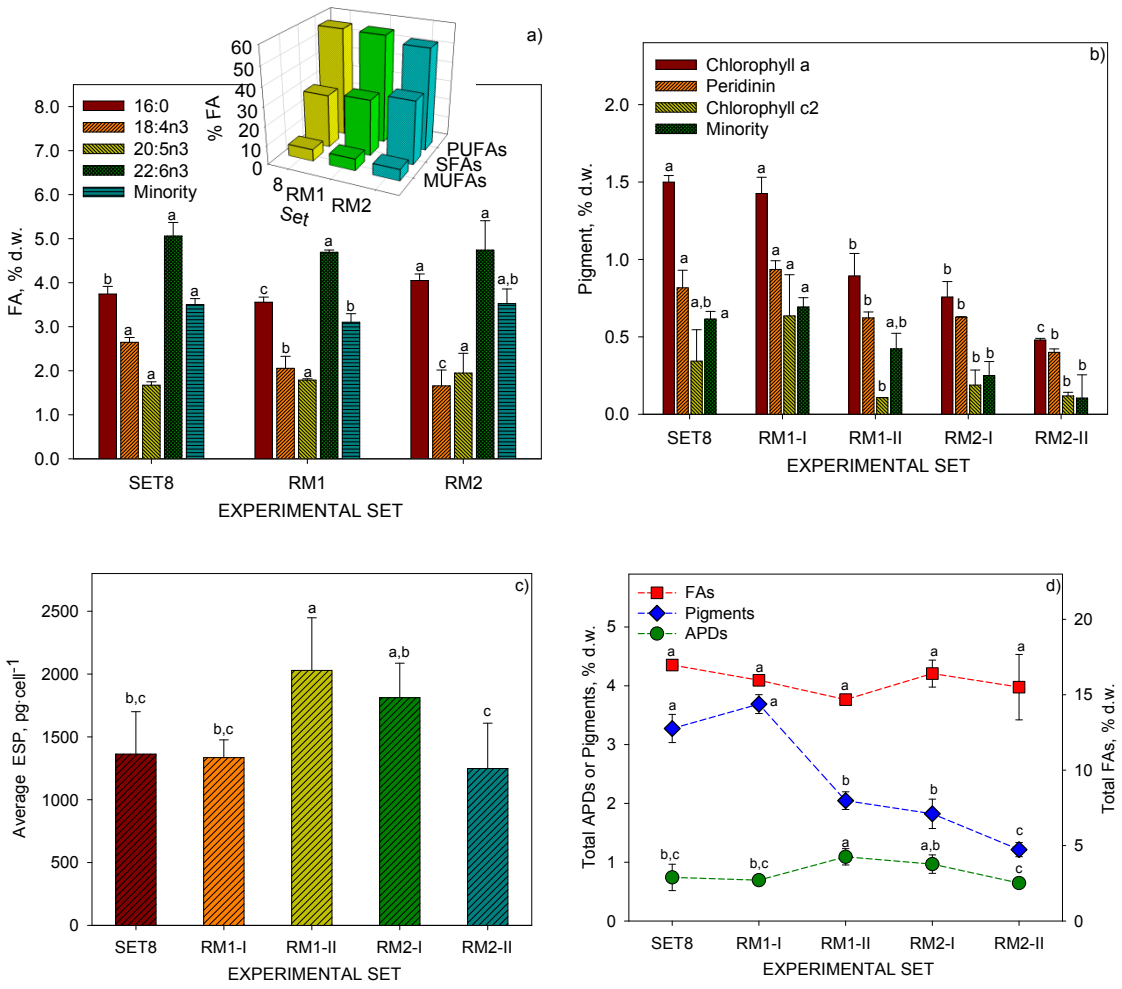


Figure 5. Characterization of the biomass of *A. carterae* averaged for values measured in the last day of the two sub-cultures in each experimental set. a) Percentage biomass dry weight for saponifiable fatty acids and distribution of the three main Fatty Acid (FA) classes (saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA)) with respect to total saponifiable FAs. These values have been averaged per set as the variability between sub-cultures was low. b) Percentage biomass dry weight for pigments. c) Equivalent saponin mass per cell (ESP). d) Percentage biomass dry weight for total saponifiable FAs, total pigments and amphidinols (APDs). Data are averages of two determinations per sample and vertical bars are standard deviations. Data with different letters between sets for each species are statistically different at the 95% confidence interval based on Fisher's least

significant difference. SET8 was carried out with fresh medium and sets RM1 and RM2 correspond to two consecutive semi-continuous cultures carried out with recycled medium. I and II refer to two consecutive sub-cultures within each set.

3.5. Bioactivity of cell extracts

The hemolytic activity of the cell extracts was used as a proxy for the APD levels in cells. Fig. 5c shows the average equivalent saponin potency (ESP) throughout the six sub-cultures. The maximum value of 2000 pg·cell^{-1} corresponds to RM1-II, followed by RM2-II, with no significant differences between the values for SET8 and RM2. The mass fraction of the APDs in the cells followed the same trend, ranging from 0.65% to 1.1% (d.w.) for the whole culture time (Fig. 5d). The biomass harvested from RM2 also exhibited strong antiproliferative activity (≤80% survival percentage; strong cytotoxic effect) against the four tumor cell lines used. This is consistent with the hemolytic activity observed due to the presence of APDs.

Although no haemolytic activities were detected in the supernatants (1 mg of extract was used in these determinations), before being discharged into the environment, the supernatant of RM2-II must be tested for the presence of APDs and/or other cytotoxic substances at trace concentrations. As such, antiproliferative activities were determined using methanolic extracts of lyophilized samples of cell-free supernatant from RM2-II. Antiproliferative activities were also very high, with no selectivity between cancer cells, exhibiting 79.7%, 78.4%, 90% and 74.7% growth inhibition for HT29, A549, MDA-MB-231 and PSN1 cell lines, respectively. As such, APDs may be released by cells into the supernatants, as reported previously for *A. carterae* [57].

4. Discussion

4.1. Acclimatisation study on a laboratory scale

The acclimatisation of inocula to specific conditions different to those in which they have been growing (e.g. modified culture medium compositions) is mandatory to obtain robust culture results. Acclimatisation is a time-dependent and species-specific process. In addition, the impact of conditions imposed on growth dynamics depends on the magnitude and direction of the shifts performed (e.g. type of illumination, nutrient source and concentration, etc.) [61].

Although RM culture from stationary growth phases might result in the accumulation of inhibitory substances [13], this was not the case here, as shown by the positive effect of the recycled medium on culture performance (see Data in Table 1). In this regard, parameters such as F_V/F_M and *SS* clearly indicated healthy dinoflagellate cells in all sub-cultures, as previously reported for dinoflagellates [55]. The spent medium could therefore be used at least four times for sub-culturing *A. carterae* without any evident deleterious effects. This observation is consistent with previous results obtained in the same RW-PBR, where repeated sub-cultures were carried out by replacing different percentages of culture broth with fresh medium without any adverse effect [3]. *A. carterae* has not been reported to produce autoinhibitory compounds. Indeed, the stimulatory effect disappeared when RM was passed through a C18 column.

The decrease in *FL*1,2,3 indicates a lower pigment content in cells compared to CTRL. This reduction was proportional to the percentage of RM used, and the values did not stabilize even after four sub-cultures. As spent medium had a TOC concentration of around 55 $mg \cdot L^{-1}$, the initial TOC concentration available to cells for the cultures ranged from 0.34 to 1.38 ng $cell^{-1}$, corresponding to 25% and 100% RM, respectively. This carbon availability may have stimulated mixotrophic growth of *A. carterae*, as usually occurs with photoautotrophic dinoflagellates [42] and microalgae [13] in the presence of TOC. With some exceptions, mixotrophically grown microalgae produce much lower

levels of chlorophylls compared to photoautotrophic conditions as less energy is needed from light [62] and the organic compounds taken up are usually the final products of photosynthesis [63]. The reduction of Chl-a cell quota could be caused, although not exclusively, by mixotrophic growth, as has also been reported for other dinoflagellate and non-dinoflagellate microalgae grown [64,65,66]. Secondary carotenoid production is barely affected at low irradiances [67].

Culture of non-dinoflagellate microalgae with recycled medium presents a wealth of cell responses [9,26,35,68,69], which mainly depend on the species [13,27], the number of times the medium is reused and replenished [13]. In some cases, decreased growth due to accumulation of inhibitors in the culture can be overcome by performing the cultures under optimal conditions [32]. However, a positive effect of the recycled medium on cell growth was generally observed. The release of growth stimulant substances and mixotrophy based on DOM generated by the cells may explain this [13,34,40,62]. Despite this, however, the information reported in the literature regarding medium recycling in dinoflagellates cultures is scarce and not significant. For example, in laboratory experiments with *A. carterae*, Dixon and Syrett [30] did not observe any effect of recycled f/2 medium on cell growth, although they reported that this medium needed to be supplemented with nutrients before reinoculation. These authors recycled 100% of the spent medium, which might explain the lack of growth improvement. In other cases, the recycled medium contains algogenic organic material (AOM) that inhibits growth [18].

The results in Fig. 1 clearly show a marked increase in P_{cell} and μ_{max} in the RM sub-cultures. The effect on pigments (analyzed using the *FL*1,2,3 values in Table 1) appears to indicate the mixotrophic metabolism of TOC as the most likely cause, as seen in other dinoflagellates [69]. To determine whether DOM was responsible for the

growth promotion observed in Fig. 2, the recirculated medium was passed through a C18 column prior to culture to remove the dissolved DOM [18]. As can be seen in Fig. 2, most of the growth-promoting species present in the supernatant appear to be retained on the C18 column as most of the stimulant effect disappears.

The study of acclimatisation in flasks based on the long-term reuse of spent medium was successfully accomplished without any quantifiable cell damage or metabolic inhibition. This strategy allowed us to envisage that complete reuse of the spent medium in the RW-PBR was feasible with *A. carterae*, at least for four sub-cultures.

4.2. Influence of medium recycling on culture performance in the RW-PBR

Fig. 3a shows a long-term, stable and robust culture of *A. carterae* in a traditional RW-PBR. The recycled medium supplemented with inorganic nutrients (sets RM1 and RM2) did not affect N_{max}, but increased both P_{cell} and P_b compared with the semi-continuous culture performed with fresh medium (SET8) (Table 2). Nevertheless, the average N_{max} was more than twice the maximum obtained with this species by Dixon and Syrett using nutrient-supplemented recycled medium [30]. Moreover, the P_b and N_{max} values were around threefold higher than those for another *A. carterae* strain cultured in a pilot-scale bubble column [57]. As observed in the small-scale acclimatisation study, there were no growth lag phases in the *RW-PBR*, and culture evolutions were very similar between sets, which could be a consequence of replenishing consumed nutrients [15,16,18]. The F_V/F_M values, which were in the range of values typically found for healthy *A. carterae* cells in the three sets [55], confirmed the absence of inhibition or toxicity [26,31]. In addition, the variation pattern for α, and thus for κ, was very similar throughout the whole culture period. As expected, α began to decrease a few days after the beginning of each batch, when nutrients clearly began to

limit growth (Fig. 3c). This is because the absorption cross-sections of microalgae strongly decrease upon nitrogen limitation of growth by modifying the pigment packaging in cells and/or the abundance of accessory light-harvesting pigments [70]. Growth was not light-limited since the average irradiance available for the cells (I_{av}) was always higher than the photosynthesis saturation value for this species (56 $\mu E \cdot m^{-2} \cdot s^{-1}$) [57].

Nitrate consumption is species-specific when using recycled medium [31,39]. Indeed, it is well documented that dinoflagellates can accumulate large amounts of nitrogen inside the cells, thus allowing growth to continue even when N is depleted in the medium [71]. In addition to the nitrate accumulated in cells, they can use the proteins released in the medium as N-source to compensate the low nitrate availability and continue growing in a mixotrophic mode (Fig. 4e). This has also been observed with *Arthrospira platensis* [72]. However, proteins did not fit the cell N-requirements for biomass generation as they decreased by a maximum of 4 $mg \cdot L^{-1}$ and biomass increased by 0.3 $g \cdot L^{-1}$ in RM2-II. In mixotrophic growth, when nitrogen sources in the medium are depleted, photosynthetic microalgae can utilize internal cell substances, such as chlorophylls, as nitrogen sources for cell division [72].

The presence of DOM in the recycled medium seemed to stimulate mixotrophy in cells and thus improve the culture performance, as has been observed with other microalgae and dinoflagellates [40,74]. Acclimatisation to mixotrophic growth occurred during four sub-cultures, as SCR_{TOC} increased to a maximum in RM2. It is well documented that the presence of organic carbon in the medium stimulates mixotrophy [40,44], which in turn increases growth rates and biomass productivities [10,40]. As HSs were clearly the majority species in the DOM, it is feasible that they are chiefly responsible for the stimulating effects. HSs are a source of both organic carbon and

nitrogen. Although the source of HSs in microalgae cultures remains unclear, decomposition of cell debris may be responsible [29]. *Amphidinium* species are naturally found in sand sediments [74], where organic matter, including HSs, is abundant and photoautotrophic *Amphidinium* strains have been found to be mixotrophs [75]. HSs have also been reported to be growth stimulating compounds for many photoautotrophic dinoflagellates [42] as they can act as ion transporters [29] and can also be metabolized as organic substrates in hetero- and mixotrophic growth [25]. However, HSs can also inhibit growth in a species-specific manner [76].

The improvement in P_{cell} for RM1 and RM2 relative to SET8 was only comparable to that observed for the 25% RM experiment on a small scale (Fig. 2), probably because the initial cell concentrations were about 20 times higher in the RW-PBR than in the small-scale assays ($8 \cdot 10^5$ *vs* $4 \cdot 10^4$ cells·mL^{-1}). This aspect shortened the duration of the exponential growth phase and lowered growth rates (μ_{max} of 0.34 *vs* 0.53 day^{-1}). In addition, initial light, nutrient and TOC cell ratios were lower (~0.05 ng C·cell^{-1}, in other words about eightfold lower than the 25% RM value on a small scale). As such, the cultures in the RW-PBR were N-limited and this regulated the maximum cell concentration. Thus, N_{max} was very similar in all sets and close to the maximum reported previously for this culture system [3]. Acclimatisation to the recycled medium resulted in a slight increase in cell volume (Table 2), which increased PBR biomass productivities and biomass yields relative to N and P.

To the best of our knowledge, this is the first time that a long-term (>70 days) culture of a dinoflagellate microalgae such as *A. carterae* has been performed using recycled medium, thus resulting in both higher cell and biomass productivities compared with fresh medium. Therefore, as suggested by life cycle assessments (LCAs) [10,14,17,20,77], important quantities of water and nutrients can be saved by recycling

the growth medium, thus resulting in a promising culture strategy for obtaining economically efficient and environmentally sustainable *A. carterae* biomass on a large scale. However, if the spent medium is to be discharged into the environment, it must be treated adequately to remove the DOM. This may be a general strategy for microalgae cultures to prevent eutrophication [10,23] or growth inhibition [18] in natural waters, but should be mandatory for species that release bioactive substances into the supernatant, as is the case for dinoflagellates.

4.3. Influence of medium recycling on the supernatant composition in the RW-PBR

Relatively large amounts of SOM accumulate in the RM cultures. In sets RM1 and RM2, for example, the maximum TOC concentrations were around 100 $mg \cdot L^{-1}$, which is similar to the value of 70 $mg \cdot L^{-1}$ typically reported for microalgae [22,23,26]. The evolution of TOC with culture time is also typical of DOM in microalgae cultures, with similar trends being observed for the genera *Tetraselmis* [40], *Anabaena*, *Microcystis* and *Scenedesmus* [78]. The *SAR*s for TOC, proteins and, although to a lesser extent, HSs decreased with time as ARs tended to stabilize in stationary phases, as is the case for other microalgae [79]. Relatively large amounts of HSs accumulated in a sub-culture-related fashion (Fig. 4c). This is probably related to the benthic nature of *A. carterae* [74]. Although the maximum HSs concentrations determined in this work are twice the concentration that inhibited the growth of *Alexandrium tamarense* [29], no negative effect was observed with *A. carterae*.

Proteins and very minor quantities of carbohydrates accumulated to a lesser extent. This contrasts with the typical behavior of planktonic phototrophs [22,26,27,[26]51], for which CH can represent 23-80% of the TOC in the supernatant [28]. CH excretion may be related to P-limitation (high N:P ratio) [80], although this is

not the case in the present study (N:P = 5). In contrast, the DOM composition in the supernatant was closer to that typically found for aquatic systems [81].

The presence of N-sources in the medium is necessary for carbon assimilation by microalgae cells [82], and if nitrogen limits growth (high C:N in the medium), DOM starts to be generated [32]. Thus, it was expected that TOC and, especially, HSs would start to accumulate in the medium, usually at high *AR*s and *SAR*s in sets RM1 and RM2 (Figs. 3c and 4a,b) once nitrogen started to limit growth. At the onset of the cultures, when C:N is low, protein release into the medium is stimulated, as was seen previously in other microalgae [28,79]. Cells released higher amounts of proteins in sets RM1 and RM2 compared with SET8 (Figs. 3c and 4a), in which the TOC concentration is lower. In particular, in RM2-II, when nitrate is consumed more rapidly, protein release is the highest for all cultures (highest SAR) (Fig. 4f).

4.4. Influence of medium recycling on the composition of A. carterae biomass obtained in the RW-PBR

4.4.1. Biomass elemental composition

The elemental composition was affected by the recycled medium. Thus, the high carbon concentration in the spent medium stimulated C consumption (~800 fmol·cell^{-1}·day^{-1}) and accumulation in the biomass at the expense of N and P (increasing C:N and C:P ratios from SET8 to RM2) (Table 3). This C accumulation also increased the N and P biomass yields, as more biomass is produced per mol of these elements (Table 2). Nitrate-N in the supernatants was exhausted before phosphates even though nitrates were apparently in excess in the medium (N:P = 5). Nutritional modes, culture conditions and phylogenetic differences are sources of variability for microalgae biomass elemental compositions, and significant variations in these ratios from the

Redfield ratio (C:N:P = 160:16:1) [73] have been reported for dinoflagellates (C:P=36-166, C:N= 5-11.3 and N:P=5.5-23) [83]. In the present study, the only difference between sets was the use of a recycled medium with a relatively high organic load. This, apparently, induces a stronger mixotrophic growth, which has been observed to induce C:N imbalance in the biomass compared to pure photoautotrophic growth in microalgae [72].

4.4.2. Saponifiable fatty acids

The FA profile of the *A. carterae* biomass is in line with data previously reported previously for the same species grown photoautotrophically [84,85]. Medium recycling is sometimes used as a strategy to increase FA production in microalgae as it contains a wide variety of organic compounds that promote FA synthesis [12]. However, with an average of 15.9% (d.w.) and no significant differences between sub-cultures, this was not the case for *A. carterae*. The values presented here are 55% higher than those reported for this species by Mansour et al. [85]. The FA classes were also different to the values in the work of Mansour et al., as SFAs represented an average 19% of all FAs in their experiments while the value in this work was around 30%. This high SFA value occurred at the expense of a lower PUFA content (55% vs 78%). This difference might be caused by the different culture systems (Erlenmeyer *vs* RW-PBR), growth medium (f medium vs f/2 × 3) and intraspecies variability between two different strains.

Structural FAs (PUFAs) and reserve FAs (SFAs) followed an inverse evolution from SET8 to RM2, although the change was less than 5%. This variation could be a consequence of N-limited mixotrophic growth, as nitrate was already consumed before the stationary growth phase. Accumulation of reserve substances (mainly SFAs and carbohydrates) in microalgae is a typical response to growth limitation [16,86–88].

However, FA profiles in dinoflagellates are less influenced by medium composition than in other microalgae [86].

EPA and DHA, the main structural FAs in the chloroplast, can be affected by light limitation or inhibition of the photosynthetic apparatus [89,90]. As I_{av} and F_V/F_M values were virtually the same between sub-cultures (Figs. 3a,b), variations in the cellular content of these FAs were not expected. The percentage of PA, which is the main SFA, increased slightly from SET8 to RM2 (Fig. 5a), probably as a result of mixotrophic growth. The maximum average PUFA and DHA yields of 3.98 and 2.90 mg L^{-1}, respectively, were very close to the best values obtained in the previous sets [3]. *A. carterae* has been postulated as a DHA source. As such, the results presented herein are of particular importance since this species has been shown to withstand several medium recycles without any measurable effect on DHA production over a very long culture period, thus making this strain a robust DHA source. The DHA content (5%) is very similar to the values obtained with *Aurantiochytrium* sp. In heterotrophic culture [91], although in *A. carterae* it is a high-value sub-product of APDs.

4.4.3. Pigments

Fig. 5b displays the pigment percentage by biomass dry weight for experimental sets 8 to RM2. The pigments identified, with Chl-a as majority and Peridinin (Per) as the main carotenoid, are consistent with the pigment profile for Dinophyta [92]. Per is a primary carotenoid present only in dinophytes, and is the majority carotenoid in *A. carterae* [93]. It is a light-harvesting carotenoid associated with Chl-a in a protein-based complex [94]. The Per/Chl-a ratios in this work (0.5–83) fall within the range reported for this species [95].

Medium recycling influenced pigment contents, with a progressive reduction being observed during the acclimatisation process. Thus, the majority pigments (Chl-a,

Per and Chl-c2) declined markedly from SET8 to RM2, with Chl-a being most affected. In general, mixotrophically grown microalgae present lower levels of chlorophylls compared to photoautotrophic conditions [31,72], whereas carotenoid production is hardly affected at low irradiances [67]. In addition, the intracellular concentration of N-containing pigments (Chl-a and Chl-c) has been observed to decrease in cultures in batch mode in the event of a deficiency of nitrogen and phosphorus [82,96]. Under these circumstances, cells utilize those pigments as an alternative N-source to maintain cell division, especially during mixotrophic growth [72]. As mentioned above, once nitrates have been totally consumed, extracellular proteins may not provide sufficient nitrogen and cells might use chlorophylls as alternative N sources. On occasions, a reduction in the chlorophyll content is concomitant with a change in the structural arrangements of chloroplasts, thus affecting the performance of the photosynthetic apparatus [65] and α values [72]. F_V/F_M and α did not differ significantly between sets (Fig 2a,b), therefore the efficiency of photosystems was not appreciably affected. Photoprotective carotenoids are not usually reduced under mixotrophic growth [72]. Although Peridinine might not have been used as alternative N-source, its cell concentration is linked to the Chl-a content and, therefore, less of this pigment may have been generated [66,96].

As there are no alternative, natural non-microalgal sources of peridinin [3], *A. carterae* has been recommended as a source of Per for the production of standards [92]. The Per content achieved herein ranged from 0.4% to 0.94% of biomass dry weight, which is higher than the value (0.24%) reported by Johansen et al. (1974) for this species [93]. However, it falls within the ranges reported for other dinoflagellates [93,97]. The maximum peridinin volumetric yield (5.6 mg·L^{-1}), which was obtained in RM1I, is similar to the value of 4.4 mg·L^{-1} obtained previously for this strain in this

PBR [3]. As Per is a by-product of the extraction-purification of APD processed from *A. carterae* biomass [4], the culture conditions may have not been optimal for its production, even though the productivities are comparable to other efficient culture systems whose main product is this carotenoid. Thus, productivity, based on the illuminated area, was 421 $mg \cdot m^{-2}$, which is similar to the value of 600 $mg \cdot m^{-2}$ obtained with the dinoflagellate *Symbiodinium voratum* cultured in a bench-scale twin-layer PBR (TL-PBR) located in a greenhouse [97], but lower than the optimized value of 1000 $mg \cdot m^{-2} \cdot$obtained with that species in an optimized TL-PBR with a 28-day culture period [98].

Medium recycling is, therefore, an attractive and economic strategy for producing this carotenoid as a high-value by-product of APD production from *A. carterae* cultures in traditional PBRs.

4.4.4. APDs

The hemolytic activity of the cell extracts was used as a proxy for the APD levels in cells. Fig. 5c suggests that the process of acclimatisation to the recycled medium affects APDs, which increase until the RM2-I sub-culture, subsequently stabilizing at levels similar to those found for the control SET8 in RM2-II (Fig. 5c). The mean stable EPS value of around 1300 pg $cell^{-1}$ is almost threefold higher than that reported for other strains of *A. carterae* [57]. These findings demonstrate that high APD productivities can be obtained from actively growing cells, which contrasts with previous reports for *A. carterae* [57]. The average biomass content of APDs was also affected by the acclimatisation process in a similar fashion to that for ESP (Figs. 5c,d). The maximum APD yield of 5.4 $mg \cdot L^{-1}$ was obtained for set RM2.

Samples from the RM2-II supernatant showed essentially no cell antiproliferative selectivity, although concentrated supernatant extracts presented strong

anticancer activity against the four cell lines used. *Amphidinium* strains have previously shown intraspecies variability in their bioactive profiles, with extracts presenting different cytotoxic and/or other bioactivities [99,100]. These results confirm the potential use of the polyketide metabolites generated by *A. carterae* in the biomedical industry.

Moreover, in view of Figs. 5c,d, stable culture in a conventional photobioreactor using recycled medium may represent a basis for the medium- to large-scale supply of these compounds under the concept of circular economy.

5. Conclusions

A. carterae cells have been successfully acclimatised to a 100% recycled medium without any measurable damage or inhibition. The organic carbon load in the spent medium stimulated mixotrophic growth, and both cell and biomass productivities were higher when using 75-100% recycled medium on a small scale than for control cultures performed with fresh medium. On a larger scale, only biomass productivities improved, whilst maximum cell concentrations were limited by nitrogen availability. As a result of nitrogen-limited mixotrophic growth, primary pigment (Chl-a, Per and Chl-c2) cell contents decreased upon using the recycled medium up to the fourth sub-culture. Fatty acids were slightly affected and APDs showed an acclimatisation response during the first two sub-cultures with the recycled medium. Both biomass and supernatant extracts showed strong antiproliferative activities against four human tumoral cell lines. This is the first study to report the long-term culture of a dinoflagellate species in a traditional raceway photobioreactor while recycling the exhausted culture medium. The robustness and stability of the 77-day long culture of *A. carterae* under the circular economy concept makes this species a good candidate for the economic production of bioactive compounds with pharmaceutical applications.

Contributions

All authors were involved in the conception and design of the study, acquisition, analysis, and interpretation of the data, and drafting of the paper. All authors agree to submission of the final version of the manuscript. A. Sánchez-Mirón takes responsibility for the integrity of the entire work and can be contacted at asmiron@ual.es.

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