New Culture Approaches for Yessotoxin Production from the Dinoflagellate Protoceratium reticulatum

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New Culture Approaches for Yessotoxin Production from the Dinoflagellate *Protoceratium reticulatum*

Juan José Gallardo Rodríguez, María del Carmen Cerón García, Francisco García Camacho, Asterio Sánchez Mirón,* El Hassan Belarbi, and Emilio Molina Grima

Department of Chemical Engineering, University of Almería, 04120 Almería, Spain

Fed-batch and perfusion cultures were carried out in a traditional glass 2-L bioreactor with the toxic dinoflagellate *Protoceratium reticulatum*. The maximum cell concentration obtained was 2.3×10^5 cell·mL^{-1}, which is almost 1 order of magnitude higher than the maximum previously referenced for this species. L1 medium was shown to be clearly deficient in nitrate and phosphate for this strain, and addition of highly concentrated aliquots of these nutrients allowed higher cell concentrations to be obtained. This species consumed high amounts of nitrate and phosphate, 2.1×10^{-3} and 2.3×10^{-4} μmol·h^{-1}·cell^{-1}, respectively. However, this consumption produced a very low number of cells compared to other classes of microalgae, indicating that this species is, like other dinoflagellates, a poor competitor in terms of utilization of inorganic nutrients. Higher production of toxins and pigments was strongly associated with cell number in the culture, with maximum values of 700 ng·mL^{-1} and 1321 μg·mL^{-1}, respectively. Most yessotoxins remained within the cells and not in the cell-free culture medium, and their production was not related to either the age of the culture or the cell growth phase.

Introduction

Marine microalgae of the family dinoflagelata (dinoflagellates) have gained importance in recent years because they are producers of potent biotoxins (*1*). Many toxic dinoflagellates form blooms called "harmful algal blooms" (HABs). Several human diseases resulting from eating contaminated seafood and direct human exposure to HABs are associated with toxic marine dinoflagellates. They include paralytic shellfish poisoning (PSP), diarrheic shellfish poisoning (DSP), ciguatera fish poisoning (CFP), neurotoxic shellfish poisoning (NSP), and azaspiracid shellfish poisoning (AZP) (*2*).

Critical research areas in HABs need to be identified in order to establish a series of reliable measures focused on safe, healthy quality food from marine resources, with a view to preventing and treating chronic diseases derived from these biotoxins. One key issue is to obtain enough certified patterns of most of the biotoxins and derivates produced by dinoflagellates. Achieving a reliable culture of dinoflagellates to produce the corresponding toxin would be useful to track toxins through the food chain, to specify the diagnostics of human and animal poisonings due to HABs, to clarify the action mode of toxins, and to develop more effective treatments. Unfortunately, at present we do not have at our disposal vaccines for poisoning from marine toxin dinoflagellates, so methods for verifying exposure will be needed.

The availability of sufficient amounts of toxins would also allow scientists to explore more efficiently the use of these toxins in the field of medicine. A recent review (*3*) outlines the main toxins derived from dinoflagellates, their mode of action, and their possible therapeutic qualities.

Dinoflagellate culture is the main source of finfish and shellfish toxins for biomedical, toxicological, and chemical research programs. However, culture of these microorganisms in bioreactors comes up against several major obstacles. Dinoflagellates have growth rates substantially lower than those of other taxa of similar size, and the cell concentrations attained are also lower, only a few thousand cells·mL^{-1} (*4*). There are photosynthetic and non-photosynthetic dinoflagellates (*5*); their main pigments are peridinin, chlorophyll *a*, and chlorophyll c_2. However, their low growth rates are not likely to be related to the efficiency of these pigments for harvesting light, since the dinoflagellate photosynthetic capacity per unit of chlorophyll *a* is not significantly different from that of diatoms. In fact, the studies published have been conducted with optically thin cultures. However, field data have demonstrated that dinoflagellate growth can easily be inhibited by turbulence, since there is a negative correlation between dinoflagellate abundance and strong winds or waves (*6, 7*). The shear stress levels that dinoflagellates may withstand are generally 1 or 2 orders of magnitude lower than those that damage most animal and plant cells (*8*−*10*).

Another physiological characteristic of dinoflagellates is their complex circadian cycle. Behavior and metabolism are simultaneously controlled by the circadian program to optimize access to irradiance and nitrate separated in the oceanic water column (*11*). Nevertheless, the same pattern is observed when cells are cultured indoors with nutrients and light in excess. The interactions of the dinoflagellate circadian system with the continuously changing environment developed inside photobioreactors must therefore be clarified.

The present work studies the growth kinetics, toxin and pigment production obtained in semicontinuous and perfusion fed-batch culture modes of the dinoflagellate *Protoceratium reticulatum*, in a classic glass stirred fermenter under continuous illumination. *P. reticulatum* is a producer of yessotoxins (YTXs), disulfated polyether toxins (Figure 1).

* To whom correspondence should be addressed. Tel: +34 950014025. Fax: +34 950015484. Email: asmiron@ual.es.

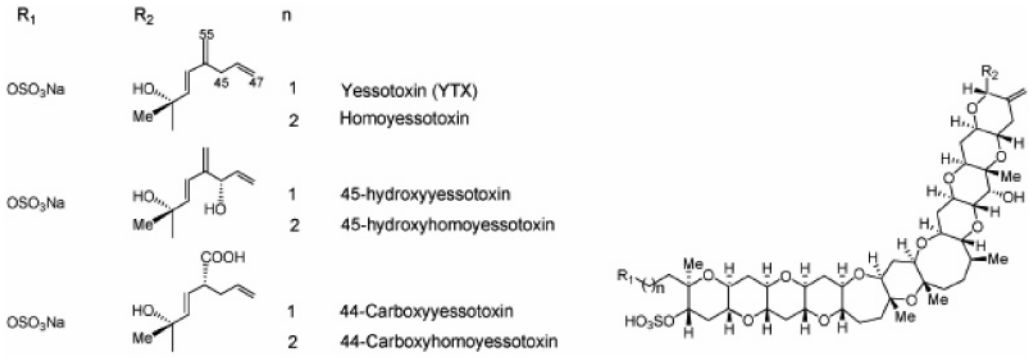

Figure 1. Yessotoxin molecule composition (*52*).

Materials and Methods

Species and Culture Medium. The species used in this work was a clone of the toxic dinoflagellate *P. reticulatum* (strain GG1AM) kindly donated by the Centro Oceanográfico de Vigo (Spain), isolated from the Mediterranean Sea and already adapted to grow in L1 medium. The medium used for the inoculum was seawater enriched according to the recipe for L1 medium (*12*). pH was fixed at 8.7 with 3 M HCl and NaOH. This medium was prepared in (0.2 μm) filter-sterilized natural seawater, and the salinity was approximately 35%. The typical composition of the Mediterranean seawater used in the cultures has been described elsewhere (*13*). The inocula were maintained in thermostatic chambers at 17 °C in static Erlenmeyer flasks with 12:12 h light:dark cycles with an average irradiance on the surface of 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ provided by 30 W cold white fluorescent lamps. Every 7 days 40% of the inocula volume was refreshed with L1 medium.

Photobioreactor. The bioreactor used was a 2-L conventional stirred glass fermenter (Braun Biotech, Germany), similar to others previously used in microalgae culture (*3*, *14*). The bioreactor had a thermostatic jacket and a 3 pitched-blade marine propeller as stirrer, 5.3 cm in diameter, working at 50 rpm. Temperature, pH, and dissolved oxygen (DO) were measured online by the control unit (Biostat B-DCU, Braun Biotech) and transmitted to a computer, where data were recorded.

Two of the experiments were run in semicontinuous mode and the other one in perfusion fed-batch mode. In the two semicontinuous experiments equal volumes of culture and fresh medium were removed and added, respectively. Since the number of cells determined in each count does not correspond to the total cells produced in the culture, the concentrations determined in every sample taken from the reactor are called "apparent cell concentrations". In the first semicontinuous experiment, after the 15th day of culture, when cell growth stopped, the strategy was changed. Small volumes of highly concentrated aliquots of nitrate (0.883 $mol \cdot L^{-1}$) and phosphate (0.0363 $mol \cdot L^{-1}$) were added along with the L1 medium in order to reach nutrient concentrations in the culture higher than those of the L1 medium. In the second semicontinuous experiment the culture strategy was always the latter. The perfusion experiment was carried out by removing cell-free culture medium using a 10 μm pore diameter polycarbonate internal spin-filter, fixed to the stirrer shaft, and operated only in fed-batch mode, i.e., cells were retained in the reactor. As the two cultivation strategies are very different, in order to compare the results obtained in the three experiments, we have calculated the total cells produced in the semicontinuous cultures taking into account the cells removed in the culture dilutions. Thus, the following equation has been used:

$$\text{total cells } (n) = \frac{[\text{cell}]_n \cdot V + \sum_{n=1}^{n} (V_{Rn} \cdot [\text{cell}]_{Rn})}{V + \sum_{n=1}^{n} V_{Rn}} \quad (1)$$

where total cells (n) is the total number of cells produced in the culture up to the nth dilution, V is the culture volume in the reactor, $[\text{cell}]_n$ is the cell concentration in the reactor after nth dilution, V_{Rn} is the volume removed from the reactor at nth dilution and $[\text{cell}]_{Rn}$ is the cell concentration in the volume removed (i.e., before adding fresh medium to the reactor). In this way, we can compare the real productivity of the semicontinuous cultures with that of the discontinuous perfusion mode.

pH was controlled by CO_2 injection on demand in the air flow, which was supplied only through the photobioreactor headspace in order to prevent bubble-associated damage to the cells. Light was applied continuously with 6 W cold white fluorescent lamps on the reactor periphery. The irradiance was measured by a 4π sensor (Biospherical Instruments Inc.) in the center of the reactor, according to previous works (*3*, *15*, *16*), and controlled by changing the number of fluorescent lamps around the reactor and/or their distance from the reactor surface. For growth measurements, samples were collected daily and fixed with Lugol's solution (*17*). The optimum proportion between fixing solution and culture was 2:100. Cell counts were taken in a light microscope using a Sedgewick rafter chamber.

Determination of Phosphate and Nitrate Concentrations. In this work only the phosphorus species that respond to colorimetric methods are determined and quantified as PO_4^{-3}, according to the method published by the American Public Health Association (*18*). Thus, molybdenic acid and ammonia-potassium tartrate react with orthophosphate in acid solutions to produce phosphomolybdenic acid, which is reduced by ascorbic acid, developing a blue color that allows the spectrophotometric quantitative analysis of phosphate at 885 nm. A 20 μL portion of the combined reactant was added to 1 mL of the supernatant sample (i.e., biomass-free culture medium) and absorbance was measured at 885 nm.

Quantitative analysis of nitrate was made by HPLC, using an array-diode detector at 210 nm wavelength, according to Moya (*19*). The column used was a Tetra-Pak C18 (4 mm × 12.5 mm) at a fixed temperature of 25 °C. The mobile phase was acidulated water (0.01 N H_2SO_4) supplied at a constant flow rate of 1 $mL \cdot min^{-1}$.

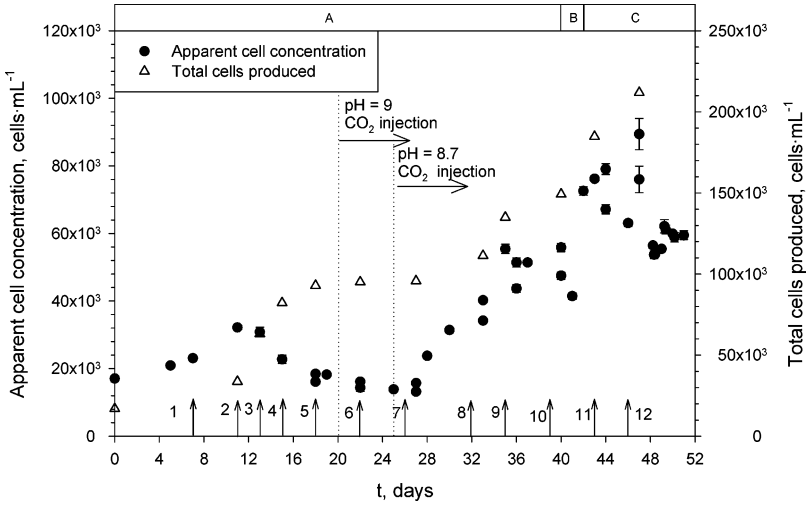

Figure 2. Evolution of the apparent concentration and total cells produced in the first experiment without the spin-filter. Arrows indicate additions to the medium and culture removals. Regions A−C at the top represent periods of different irradiance (more details are shown in Table 1).

Pigment Determination. For the determination of the pigment content in the cells, samples taken from the bioreactor were vacuum filtered through a 10 μm Whatman GF/F filter. After filtration the filter was placed in a 10 mL tube and 3 mL of methanol was added to extract the pigment from the biomass. After at least 30 min of sonication in the dark, the mixture obtained was centrifuged for 5 min at 12000 rpm. Pigment concentrations were quantified in a fluorescent HPLC (*20*). Excitation and emission wavelengths were fixed at 488 and 525 nm, respectively. Isocratic elution was at a flow rate of 1.0 mL min^{-1} with the following mobile phases: 25% solvent A (8:2 v/v methanol/water MiliQ), 75% solvent B (1:1 v/v acetone/methanol). The column used was a Merck Lichrospher 100 RP-18 phase column (4.6 mm i.d., 125 mm, 5 μm particle size). Calibrations were made by using standard samples of chlorophyll *a* and chlorophyll *b*, purchased from Sigma Chemicals and chlorophyll *c*, fucoxanthin, and diadinoxanthin purchased from DHI.

YTX Determination. YTXs were determined in the biomass and in the supernatant following the method described by Paz et al. (*17*). Briefly, YTXs were quantified in a fluorescence HPLC system (Shidmadzu AV10) with a fluorescence detector (RF-10AX) and an autoinjector (SIL-10ADVP). The YTX molecule must first be transformed into a fluorescent molecule by reacting with DMEQ-TAD (4-[2-(6,7-dimethoxy-methyl-3-oxo-3,3-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione). A reverse phase column was used (Lichrospher 100 RP-18, 5 μm, 125 mm × 4 mm, Merck). The mobile phases were 0.1 M ammonium acetate (pH 5.8) for toxin elution and methanol/water (70:30) to elute any organic matter from the column before the next analysis. During the whole run the total flow was 0.75 mL·min^{-1}. The excitation and emission wavelengths were 370 and 440 nm, respectively.

Calculation of Specific Growth, Nutrient Consumption Rates, and YTX Specific Yield. Specific growth rate μ (d^{-1}) was calculated from the equation

$$\mu = \frac{\ln\left(\frac{N_{i+1}}{N_i}\right)}{t_{i+1} - t_i} \tag{2}$$

where N_{i+1} and N_i are the cell concentrations (cells·L^{-1}) at time t_{i+1} and t_i (d), respectively.

The nutrient uptake rates, ρ_S, (μmol·h^{-1}·$cell^{-1}$) were calculated with the equation:

$$\rho_S = \frac{S_{i+1} - S_i}{24(t_{i+1} - t_i)\bar{N}} \tag{3}$$

where S_{i+1} and S_i are the nutrient concentrations (mol·L^{-1}) at times t_{i+1} and t_i, respectively, and $\bar{N}$ is the average cell concentration over the time interval considered, since there is cell growth in the time interval of days.

We have also defined a nutrient cell yield, Y_S, (cell·d^{-1}·mol^{-1}) as

$$Y_S = \frac{N_{i+1} - N_i}{(t_{i+1} - t_i)(S_{i+1} - S_i)} \tag{4}$$

The maximum nutrient yield in YTX (ngYTX·d^{-1}·mol^{-1}) was defined as

$$Y_{YTXmax} = [\overline{YTX}]_{cell} Y_{Smax} \tag{5}$$

where $[\overline{YTX}]_{cell}$ is the average concentration of yessotoxins in the biomass (ng·$cell^{-1}$) in the whole culture run. In this equation the average toxin content in the cells was used because the frequency of the toxin analysis was lower than that of the nutrient and cell concentrations.

Results and Discussion

Semicontinuous Experiments (No Spin-Filter). ***First Experiment.*** This work presents a novel approach to the cultivation of a strain of *P. reticulatum* in a traditional stirred fermenter. It is practically impossible to control pH by CO_2 injection in inocula flasks, and the conditions in the two systems are inherently very different. A first experiment was therefore necessary in order to optimize the culture conditions as regards the three nutrients required by a photoautotrophic microorganism: light, inorganic carbon, and main inorganic nutrients (nitrates and phosphates). Therefore, the first semicontinuous experiment is intended for comparison with later assays.

Figure 2 depicts the evolution of cell concentration against time. As a typical semicontinuous culture the bioreactor was operated in batch mode during the first 11 days, until growth stopped, and later in semicontinuous mode. The first period can

Table 1. Irradiance and Culture Strategy Followed in the First Semicontinuous Experiment without Spin-Filter

irradiance ($\mu E \cdot m^{-2} \cdot s^{-1}$)	events	culture volume removed (mL)	addition of medium and nutrients[a]
(A) 34.2	1	0	2 mL stock L1
	2	100	100 mL L1
	3	100	100 mL L1
	4	100	100 mL L1
	5	100	400 mL L1
	6	300	300 mL L1
	7	320	320 mL L1 + 2 mL nitrate stock + 4 mL phosphate stock
	8	300	300 mL L1 + 2 mL nitrate stock + 4 mL phosphate stock
	9	300	300 mL L1 + 2 mL nitrate stock + 4 mL phosphate stock
(B) 44	10	300	300 mL L1 + 2 mL nitrate stock + 4 mL phosphate stock
(C) 54.5	11	300	300 mL L1 + 1 mL nitrate stock + 4 mL phosphate stock
	12	300	300 mL L1 + 1 mL nitrate stock + 4 mL phosphate stock

[a] 1 mL of stock contains nutrients equivalent to those of 1 L of L1 medium.

be used as a typical discontinuous control culture. In this period cell concentration is the lowest, and therefore light availability is the highest, with the maximum specific growth rate for a batch culture. The culture strategy for the whole experiment is explained in Table 1 and indicated with arrows in Figure 2. During the first 20 days of the culture the additions consisted of fresh L1 medium, attaining a maximum cell concentration of 3×10^4 cell·mL^{-1} on day 11 with a very low specific growth rate, 0.06 d^{-1}, and an apparent cell productivity of 1374 cell·mL^{-1}·d^{-1}.

After day 13 the cell concentration started to decline, reaching values similar to the initial ones on day 25. This decline in the cell concentration might be attributed to a washout of the culture caused by the removals of culture. During the first 20 days the phosphate concentration remained very low, never below zero, and growth stopped at day 11. In order to discard the other possible growth-limiting nutrient, inorganic carbon, on day 25 the pH was controlled at the original value of the L1 medium, 8.7, by CO_2 injection on demand. However, in order to prevent any deleterious effect of this pH change, it was first fixed at 9 on day 20. In static cultures pH is left uncontrolled, but because of the mobility of the cells and the high surface-to-volume ratio of the Erlenmeyer flasks, they can swim to the gas−liquid interface to obtain this nutrient. There is a low surface-to-volume ratio in the reactor, and cells cannot swim freely due to the mechanical agitation. For these reasons it was necessary to ensure that the inorganic carbon was not limiting cell growth by injecting CO_2 on demand. As shown in Figure 2, on day 25 the cell concentration remained almost constant, which means that at these low cell concentrations the CO_2 was not a growth-limiting factor. Thereafter, the limiting nutrients could only be the inorganic salts, since in small-scale static cultures (data not shown) higher cell concentrations were attained with lower light availability and higher nitrate and phosphate concentrations. Therefore, the strategy of nutrient addition was changed, opting to refresh high volumes of culture and increase the concentration of nitrate and phosphate, the other possible limiting nutrients according to previous results (*5*) and the experience in static cultures (data not shown). Though the volumes removed were three times the previous ones (300 mL), this strategy produced a marked increase in the cell concentration, with an apparent specific growth rate of 0.14 d^{-1}, which is similar to other species with low growth rates, such as *Prorocentrum lima* (*21*), and an average cell productivity of 4157 cell·mL^{-1}·d^{-1}. This tendency continued until day 38, when cell concentration tended to stabilize at a value of 6×10^4 cell·mL^{-1}. At this point, day 41, since cell concentration was very high and growth limitation by other nutrients had been discarded, irradiance was increased by 22% and cell concentration increased, until day 43, in approximately the same proportion to 8×10^4 cell·mL^{-1}. Further increments in the irradiance caused a cell concentration of about 9×10^4 cell·mL^{-1}, which improves on the maximum values reported for this species 8-fold (*17*), 6-fold (*22*), and 2-fold (*23*). *P. reticulatum* has a very low specific growth rate compared to other species (*24*), and despite the continuous illumination and stirring, which broke the dinoflagellate cells' circadian rhythm, the strain used in the present experiment has proved capable of maintaining long-term growth and obtaining the highest cell concentrations ever referenced.

The amount of cells generated in the culture was extremely high, since the culture volumes removed also contained a high number of cells. Taking into account the cells generated per volume unit (eq 1), Figure 2 shows that the real maximum concentration of cells produced in this culture was almost 2.4 times higher than the apparent one (cell concentration determined in the reactor at any sampling time) (2.12×10^5 versus 8.94×10^4 cells·mL^{-1}) and between 5 and 15 times the highest ever referenced. The specific growth rates calculated (see Table 2) were 0.062 d^{-1} for the first 10 days, increasing to 0.31 d^{-1} from days 10 to 13, and decreasing again to 0.046 d^{-1} from days 33 to 47, but at very high cell concentrations. The cell productivities increased in similar fashion over the same time periods reaching 1521, 14683, and 7187 cell·mL^{-1}·d^{-1}, respectively, for the mentioned periods, values that are much higher than the apparent ones obtained.

Figure 3a and b may help to understand the behavior observed in Figure 2. These figures show the values of nitrate and phosphate concentration before and after the additions of nutrients. In both cases for the first 24 days, in which the cell growth was very poor, the concentration of nutrients remained invariable at levels below those of the original L1 medium. In fact, during this time the impact of the addition of fresh medium on the nutrient concentration profile was imperceptible. In view of Figures 2 and 3 it is clear that during the first part of the experiment the culture was clearly limited by nitrate and phosphate and that when the addition strategy was changed the concentration in the medium of those nutrients increased, stabilizing at the end of the culture along with a relatively very high cell density. This nutrient accumulation after the first 24 days of culture allowed us to discard any growth limitation by inorganic nutrients in the last period, leaving light availability as the only growth-limiting nutrient. One can therefore conclude that L1 medium, a classic medium for the culture of dinoflagellates, may be very poor in nitrate and phosphate and inadequate for maintaining a high cell concentration. The great consumption observed for this strain shortly after the additions is noteworthy because, in some cases, it represents roughly 50% of the nutrients added, despite their accumulation in the supernatant.

Although the main goal of this work was not to study the short-term nutrient uptake kinetics, the long-term nutrient

Table 2. Cell Productivity and Specific Growth Rates Calculated in the Three Cultures[a]

first semicontinuous						second semicontinuous						perfusion		
apparent			total			apparent			total			total		
t	P_b	μ	t	P_b	μ	t	P_b	μ	t	P_b	μ	t	P_b	μ
0−11	1374	0.06	0−11	1521	0.06	0−16	2838	0.11	0−14	3673		0−20	7050	0.14
25−35	4157	0.14	11−13	14683	0.313	26−30	6192	0.14	14−19	6872	0.09			
			25−33	3068	0.027	31−43	1440	0.02	47−63	2219	0.016			
			33−47	7187	0.046									

[a] t = period at which the variables are evaluated, days; P_b = cell productivities, cell·mL·d^{-1}; μ = specific growth rate, d^{-1}.

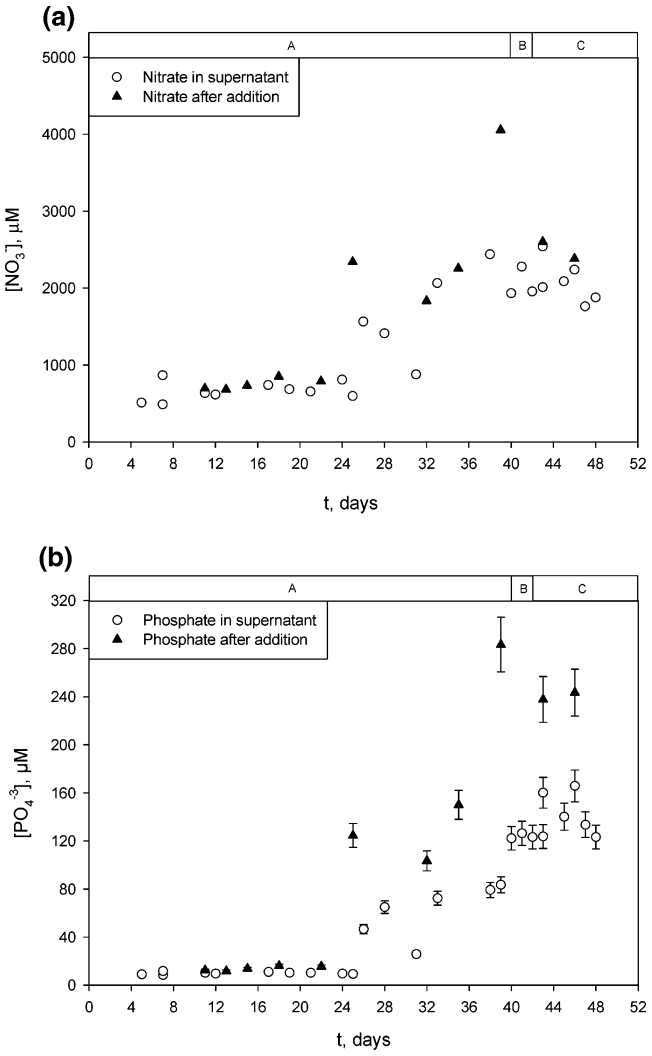

Figure 3. Variation of nutrient concentration with time in the first experiment without spin-filter: (a) nitrate; (b) phosphate.

specific consumption rates (ρ_S) and their corresponding biomass yields (Y_S) can be estimated from eqs 4 and 5. The values of these variables, along with the cell concentration and time at which they were calculated, are shown in Table 3. There is not a clear and unique relationship between ρ_S and Y_S with time or cell concentration, but it seems clear that the higher the nitrate and phosphate concentration the higher the specific consumption rates. Thus, the highest nitrate consumption rate of 1.61×10^{-3} μmol·cell^{-1}·h^{-1} was achieved at day 39, followed by days 26, 28 and 41 with 1.1×10^{-3}, 3.32×10^{-4} and 2.36×10^{-4} μmol·cell^{-1}·h^{-1}, respectively. Overall, cell yield followed an inverse evolution, i.e., the higher the consumption rate the lower the cell production per micromole of nutrient. This may imply a fruitless use of nitrate or an internal accumulation of the nutrient in an intracellular pool for later consumption as observed by Lomas and Glibert (*25*). The low cell yield of

Table 3. Nutrient Consumption Rate (ρ_S), Cell Yield (Y_S) for Nitrate and Phosphate in the Three Experiments, and Maximum Nutrient Toxin Yield[a]

		1st expt (no spin-filter)		2nd expt (no spin-filter)		expt with spin-filter	
nutrient	parameters	value	t (d)	value	t (d)	value	t (d)
NO_3^-	$10^3 \times \rho_{max}$	1.61	39	2.08	19	0.71	22
	$10^5 \times \rho_{min}$	2.26	5	0.13	58	0.495	37
	$10^4 \times \rho_{av}$	3.17		2.61		2.51	
	Y_{max}	45.80	5	56.91	21	206.29	47
	Y_{min}	0.85	39	10.95	7	3.27	1
	Y_{av}	13.53		28.52		40.16	
	Y_{YTXmax}			227.64	33	736.46	47
PO_4^{-3}	$10^5 \times \rho_{max}$	22.9	25	8.66	7	2.48	16
	$10^7 \times \rho_{min}$	4.86	5	11.4	40	4.12	23
	$10^5 \times \rho_{av}$	2.71		1.75		1.52	
	Y_{max}	2127.16	5	494.57	12	5325.14	23
	Y_{min}	3.72	39	74.80	40	5.82	30
	Y_{av}	426.77		290.54		256.41	
	Y_{YTXmax}			1162.16	33	19010.75	13

[a] ρ_{max}, ρ_{min}, and ρ_{av} represent maximum, minimum, and average nutrient uptake rates, respectively (μmol·h^{-1}·cell^{-1}). Y_{max}, Y_{min}, and Y_{av} represent maximum, minimum, and average nutrient cell yield, respectively (cells·μmol^{-1}·d^{-1}). Y_{YTXmax} represent the maximum nutrient toxin yield (ngYTX·μmol^{-1}·d^{-1}).

nitrate indicates that this species has a behavior similar to that of other dinoflagellates (*26*), confirming that dinoflagellates are not good nitrate competitors compared to other marine species.

The evolution of phosphate consumption was similar, with a maximum of 2.29×10^{-4} μmol·cell^{-1}·h^{-1} at day 25, when the concentration of this nutrient is close to the maximum, followed by day 39 (1.22×10^{-4} μmol·cell^{-1}·h^{-1}). For both nutrients, the highest yields coincided with the lowest concentrations, i.e., at the beginning of the culture, at day 5 (45.8 cell·μmol^{-1}·d^{-1} for nitrate and 2127 cell·μmol^{-1}·d^{-1} for phosphate). When cells are almost starved their metabolism seems highly efficient, producing a relatively high cell density with a very low nutrient consumption. However, despite the higher nitrate consumption, cell yields are 1 order of magnitude higher for phosphate, indicating that if there is an internal pool for phosphate, it is less important in these microorganisms, or that the anabolism for this nutrient is more efficient than for nitrate. It is clear that cells increase the uptake of nutrients when the growth and the cell concentration are high, except in the stationary phase; both growth and cell density are enhanced by the additions of concentrated nutrients in the semicontinuous culture approach used. This behavior has also been reported for the dinoflagellate *Gymnodinium catenatum* (*26*), whose uptake of nitrate and phosphate increased with the concentration in the medium. Its short-term maximum consumption was 6.48×10^{-6} μmol·cell^{-1}·h^{-1} for nitrate and 1.42×10^{-6} μmol·cell^{-1}·h^{-1} for phosphate (*26*). These values are lower than the maxima obtained in the present work. Cell concentrations were also very low compared to those presented here, and the nutrient concentrations tested in those experiments were about 10 times lower for nitrate and 5 times

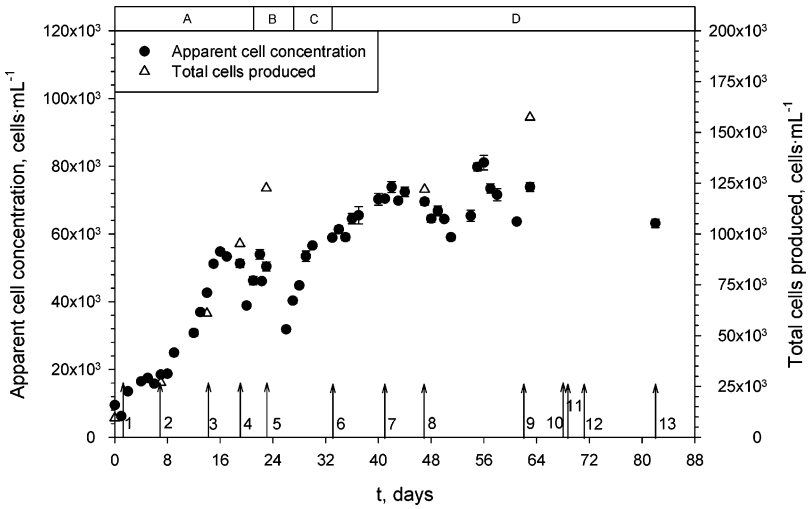

Figure 4. Evolution of the apparent concentration and total cells produced in the second experiment without the spin-filter. Arrows indicate additions to the medium and culture removals. Regions A−D at the top represent periods of different irradiance (more details are shown in Table 4).

lower for phosphate. Even lower values have been reported (*27*) (1.2×10^{-6} μmol·cell^{-1}·h^{-1}) for phosphate consumption with *Alexandrium tamarense* under lower phosphate concentrations.

Second Experiment. In order to clarify the causes of the limited growth shown in the first 24 days of the previous culture, a second experiment was carried out using the renewal strategy of the last part of the previous culture from the outset. Thus, the results shown in Figure 4 were obtained under the conditions listed in Table 4. As expected, this culture presented faster initial growth, with an apparent maximum specific growth rate of 0.11 d^{-1} and a productivity of 2834 cells·mL^{-1}·d^{-1}, from the beginning of the experiment until day 16; see Table 2. As the irradiance in the center of the reactor in the first region of this experiment (zone A) was very similar to that of the first experiment (32.5 μE·m^{-2}·s^{-1}, Table 1), the cell concentration stabilized at a very similar value, 6×10^4 cells·mL^{-1}. This behavior demonstrates that the culture at that irradiance was light-limited, that an increase in the irradiance would increase the cell concentration, and that the reproducibility of the data, despite the difference in the inocula, is very good. In fact, when irradiance was increased in regions B, C, and D, the cell concentration again increased to values of over 8×10^4 cell·mL^{-1}. When the irradiance was changed, the cell health was checked by following the DO evolution, since this variable is a fast indicator of photoinhibition processes (*28*−*31*). If a decrease in DO was observed in the hours after the irradiance step, it was reduced to a value in which the DO value was higher than the initial one. In this way we were always sure that the irradiance levels never induced photoinhibition.

The maximum specific growth rate took place in region B (between days 26 and 30), with a value of 0.144 d$^{-1.}$ In any case, in this experiment cells maintained their growth over a longer period of time with the irradiance increments. In region B the cell productivity was 6192 cells·mL^{-1}·d^{-1}, which was higher than the maximum obtained in the previous experiment. Taking into account the cells removed very high concentrations of about 16×10^4 cells·mL^{-1} were attained (Figure 4). Although the period of maximum growth rate was in the first 14 days with a value of 0.133 d^{-1}, which is similar to the apparent growth rate and to the one obtained in the previous semicontinuous experiment, maximum productivity (see Table 2) was 6872 cells·mL^{-1}·d^{-1} between days 14 and 19. Since the two experiments start from different inocula, the similar behavior and growth patterns demonstrate the robustness of the culture system and strategy followed.

Figure 5 shows that nitrate (a) and phosphate (b) were gradually accumulated during the first 24 days and then a plateau was attained for nitrate, with a slight increase for phosphate. The accumulation shown means that the cell growth was not limited by these salts and that the only limitation could come from light, since cell concentration increased with irradiance. In this case, as Table 3 shows, the nitrate uptake rate was highest for the highest concentration tested. The ρ values obtained in this second experiment were similar to those in the first one, although ρ_{max} did not take place when the nitrate concentration was maximum, i.e., on day 19 (2.08×10^{-3} μmol·cell^{-1}·h^{-1}) but on day 23 (6.03×10^{-4} μmol·cell^{-1}·h^{-1}). In this experiment, the cell yield for nitrate is higher than in the previous one, in some cases 1 order of magnitude higher (10.95 cells·μmol^{-1}·d^{-1} compared to 0.85 cells·μmol^{-1}·d^{-1} for the minimum values). This behavior was also observed on a small scale (data not shown), and it may suggest that there is a certain residual effect of limiting initial cell growth, since after the first part of the first experiment, when cells were strongly limited by nutrients, they presented greater nutrient consumptions with very low cell yield, showing a very inefficient nutrient use. In the case of phosphate, the consumption rates presented the same behavior in this experiment as in the former one. The values were similar, with a maximum of 8.7×10^{-5} μmol·cell^{-1}·h^{-1} (day 7), and a slightly lower minimum (1.14×10^{-6} μmol·cell^{-1}·h^{-1} on day 40). Cell yields were higher in the second experiment with the exception of the first few days. This fact could corroborate the more efficient metabolism of cells growing in a non-limiting environment with respect to those that have passed through a long limited growth period.

Experiment with Spin-Filter (Perfusion Culture). The main difference between this operational mode (perfusion) and the previous ones (semicontinuous) is that now the cells remain in the photobioreactor because of the spin-filter, allowing only biomass-free culture medium (supernatant) to be removed in the successive extractions.

Figure 6 shows the evolution of cell concentration when the reactor was operated in perfusion mode with the internal spin-filter. The feeding strategy is described in Table 5 and was similar to the previous experiments, i.e., the base L1 medium was enriched with nitrate and phosphate concentrated stock. The

Table 4. Irradiance and Culture Strategy Followed in Second Experiment without Spin-Filter

irradiance (μE·m^{-2}·s^{-1})	events	culture volume removed (mL)	addition of medium and nutrients[a]
(A) 32.5	1	0	2 mL stock L1
	2	300	300 mL L1 + 4 mL nitrate stock + 4 mL phosphate stock
	3	300	300 mL L1 + 4 mL nitrate stock + 8 mL phosphate stock
	4	300	300 mL L1 + 4 mL nitrate stock + 4 mL phosphate stock
(B) 56.6	5	300	340 mL L1 + 4 mL nitrate stock + 4 mL phosphate stock
(C) 81.6			
(D) 66.7	6	0	100 mL L1 + 4 mL phosphate stock
	7	0	6 mL phosphate stock
	8	40	250 mL L1 + 3 mL nitrate stock + 10 mL phosphate stock
	9	100	100 mL L1 + 3 mL nitrate stock + 10 mL phosphate stock
	10	100	100 mL L1 + 3 mL nitrate stock + 10 mL phosphate stock
	11	40	40 mL L1 (3 mL nitrate stock + 10 mL phosphate stock)
	12	100	100 mL L1 (3 mL nitrate stock + 10 mL phosphate stock)
	13	0	2 mL nitrate stock + 4 mL phosphate stock

[a] 1 mL of stock contains nutrients equivalent to those of 1 L of L1 medium.

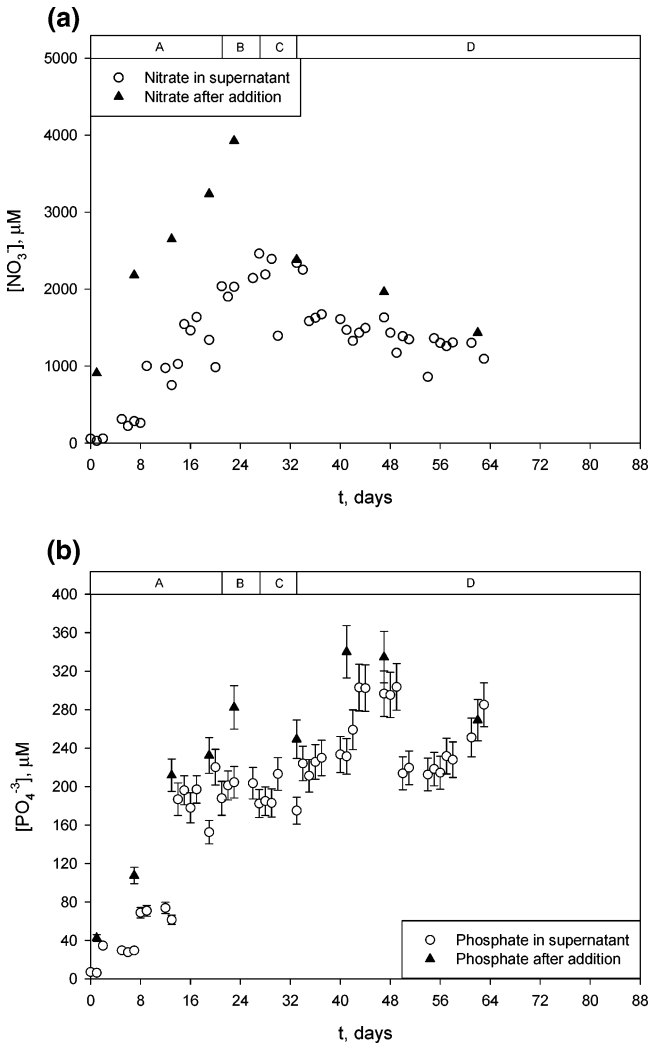

Figure 5. Variation of nutrient concentration with time in the second experiment without spin-filter: (a) nitrate; (b) phosphate.

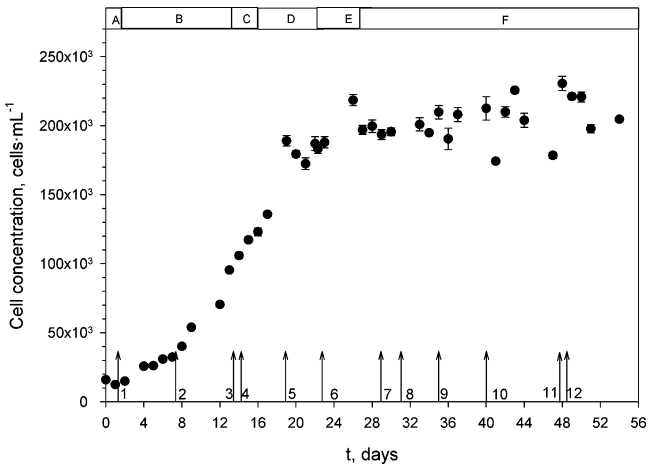

Figure 6. Evolution of cell concentration in the experiment with the internal spin-filter. Arrows indicate medium additions and removals. Regions A−F at the top represent periods of different irradiance (more details are shown in Table 5).

tendency of the cell concentration is typical for a light-limited batch culture. The cells started with a 3-day lag phase followed by an exponential growth phase with a maximum specific growth rate of 0.14 d^{-1} and an average productivity of 7050 cells·mL^{-1}·d^{-1} (see Table 2), slightly higher than the value obtained in the semicontinuous experiment, due to the higher specific growth rate.

The end of the linear growth phase (zone D) was reached on day 20 at a cell concentration of 18 × 10^4 cells·mL^{-1} and an irradiance of 83.2 μE·m^{-2}·s^{-1}. Thereafter, the irradiance was reduced to 55.1 μE·m^{-2}·s^{-1} (zone E) in order to check whether the cell growth stopped as a result of photoinhibition. As cell concentration did not significantly change, the irradiance was increased once more to the value of zone F. There was a slight increase in cell concentration in region F at maximum irradiance of 110.7 μE·m^{-2}·s^{-1}, reaching a maximum value of 23 × 10^4 cell·mL^{-1}. This value is between 5 and 15 times higher than previously referenced maxima and very similar to the accumulated cell concentration obtained in the first and second experiments without spin-filter (see the total cells accumulated in Figures 2 and 4). Most of the experiments with dinoflagellates are carried out in discontinuous mode.

Figure 7a and b shows the nitrate and phosphate concentrations versus culture time. Up to day 28 fresh medium and nutrients were added 5 times and partially accumulated, almost linearly with time. Despite this accumulation, nitrate consumption was very high shortly after each addition, around 50% of the nutrient added. After the additions the consumption rates decreased and a certain amount of nitrate was even released to the medium. Phosphate was consumed at a lower rate and accumulated during the whole culture period. From day 28 until the end, the volumes added were smaller (200 mL) and the nitrate concentration decreased slowly toward the end of the experiment. In period F, cell concentration was very high but constant, although consumption was still high, as the supply

Table 5. Irradiance and Culture Strategy Followed in Experiment with Spin-Filter

irradiance (μE·m^{-2}·s^{-1})	events	supernatant vol removed (mL)	addition of medium and nutrients[a]
(A) 32.5	1	0	2 mL stock L1
(B) 56.6	2	300	300 mL L1 + 4 mL nitrate stock + 4 mL phosphate stock
	3	300	300 mL L1 + 4 mL nitrate stock + 8 mL phosphate stock
(C) 67.6			
(D) 83.2	4	300	300 mL L1 + 4 mL nitrate stock + 4 mL phosphate stock
(E) 55.1	5	300	340 mL L1 + 4 mL nitrate stock + 4 mL phosphate stock
(F) 99.2	6	200	200 mL L1 + 0.4 mL phosphate stock
	7	200	200 mL L1 + 0.4 mL phosphate stock
	8	400	400 mL L1 + 3.2 mL nitrate stock + 6.8 mL phosphate stock
	9	2000	2000 mL L1 + 3 mL nitrate stock, 16.5 mL phosphate stock
	10	400	400 mL L1 + 3 mL nitrate stock, 16.5 mL phosphate stock
	11	85	85 mL L1 + 3 mL nitrate stock, 16.5 mL phosphate stock
	12	180	180 mL L1 + 3 mL nitrate stock, 16.5 mL phosphate stock

[a] 1 mL of stock contains nutrients equivalent to those of 1 L of L1 medium.

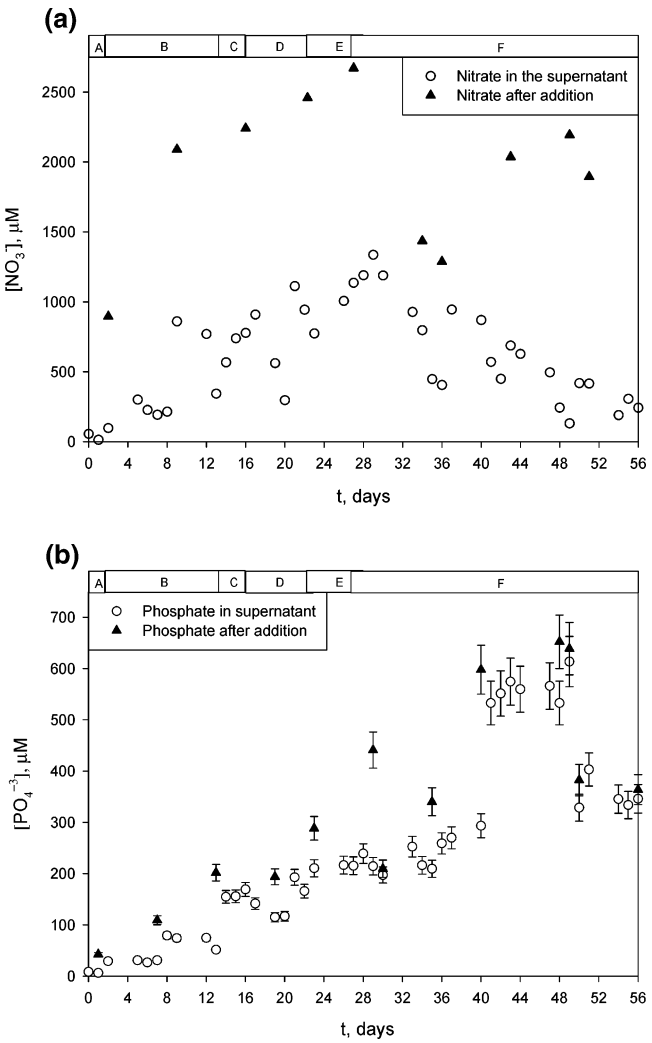

Figure 7. Variation of nutrient concentration with time in the experiment with the internal spin-filter: (a) nitrate; (b) phosphate.

was not sufficient to maintain the nitrate concentration constant. This is evidence of the high maintenance metabolism of this strain.

The results of nitrate and phosphate specific consumptions and cell yields are also displayed in Table 3. The average consumption rates (ρ_{av}) for both nutrients are similar to those of the two previous experiments, indicating that the culture method does not seem to have an appreciable influence on nutrient uptake and that cell physiology is the most important variable. The highest values took place at very high concentrations in the medium (7.1×10^{-4} μmol·cell^{-1}·h^{-1} for day 22), although the values were not as high as in the previous experiments, probably because the maximum concentrations were also lower in the semicontinuous experiments. In this culture, the average cell yields were similar to those obtained in the experiments without spin-filter. Note that in this experiment appreciable nitrate amounts were released to the medium, after peaks in consumption the nitrate concentration in the medium increased (see Figure 7a). Dinoflagellates can release reduced forms of nitrogen, nitrites and ammonium, to the medium at high rates (*32*). Since nitrites have not been detected in our HPLC analysis, the nitrate release could correspond to a non-nutritionally reduced form as a consequence of simple diffusion from the cells to the medium (*24*, *25*). Further research is therefore required in order to identify these species. Very high values have been found for K_S (substrate concentration at which the specific growth rate is half the maximum) in *Prorocentrum minimum* (*33*). These high values mean that dinoflagellates might have a very low affinity for nutrients, which is a disadvantage compared to diatoms in environments with low nitrate concentrations (i.e., the higher the K_S value, the lower the substrate consumption efficiency). A non-saturating uptake kinetic for nutrients in diatoms, probably due to simple diffusion from the medium to the cells, has been previously reported (*34*). This process could explain the high nitrate consumption rates shortly after the additions in spite of the high levels in the culture medium. The slight release of nitrate in the days following this rapid consumption, when nitrate concentration in the culture broth was strongly reduced, could be explained if a fraction of the nitrate previously absorbed leaked through the cell membrane to the medium.

Although the present work does not include a detailed study of consumption kinetics, the results obtained and previous studies (*26*, *27*) point to the fact that dinoflagellates consume higher amounts of nutrients when the concentrations are higher in the medium. The values obtained were very high, but the concentrations in the cultures were also high. This may imply that the metabolism of the macronutrients in these relatively unknown species is complex and that the higher the concentration is, the higher the consumption.

Reviewing the data reported in the literature, it is clear that no strain of *P. reticulatum* has reached such high cell concentrations as the values presented here. The maximum cell concentrations referenced are a few tens of thousands cells·mL^{-1}. With the perfusion feeding strategy we have been able to obtain values several times higher, which shows the importance of maintaining

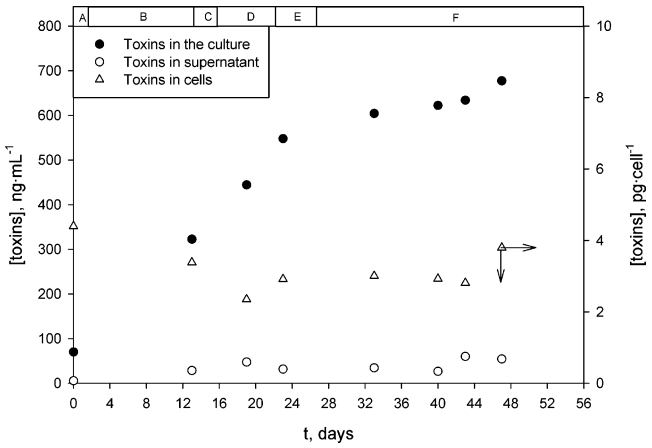

Figure 8. YTX concentration in the experiment with the internal spin-filter.

a relatively high nutrient level in the culture medium along with light increments when cell concentration stabilizes.

Furthermore, in view of the results obtained in this and other previous works, the variability in the indicative parameters of the cell nutrient requirements in dinoflagellates is considerable, and more detailed experimental work has to be done in order to clarify the uptake and the release kinetics of nutrients in this species.

Toxins. Whereas the first experiment was aimed solely at optimizing culture conditions, in the second and third ones the YTX content was determined. As an example, Figure 8 shows the cell content, the supernatant content, and the total YTX content in the perfusion culture. The supernatant content was almost constant, very low and similar in both experiments, with an average value of 30 ng·mL^{-1}. This behavior has also been reported previously (*17*). YTXs are intracellular bioproducts and their low concentration in the supernatant might be the consequence of cell lysis after the death process associated with apoptosis and/or mechanical stress. The presence of the toxins in the supernatant has also been previously attributed to cell breakage and death in dinoflagellates (*21*). However, some authors have found increased YTX concentrations in the supernatant in cultures of *P. reticulatum*, which are apparently unrelated to cell death (*35*). In our opinion these results should be considered with caution because culture procedures were briefly described and lethal events for cells could not be identified.

In both experiments (see Figure 8 as an example for the perfusion experiment), YTX contents in cells were practically constant during the culture time, around 3.57 and 4 pg·cell^{-1} for the perfusion and semicontinuous experiments respectively (see Table 6). Thus, in both cases the increase in YTX concentration was mainly associated with the number of cells and to a lesser extent with cell growth phase. Since the cell concentration in the perfusion experiment is the highest ever referenced, the toxin concentration in the culture is the highest ever attained and more than twice that attained in the semicontinuous culture (see Table 6). Some authors (*17*, *21*, *36*) have found an increase in YTX content within cells with culture age. Perhaps the differences observed with the reported results are influenced by culture method, since the aforementioned authors worked in batch mode and in the present work the experiments were carried out in fed-batch mode. More detailed data of cell diameter could have helped to explain the differences observed.

The toxin content may also be influenced by nutrient availability, which has been previously reported (*37*). These

Table 6. Average YTX Content in Supernatant and in Cells and Maximum Concentration in Culture Obtained in Semicontinuous and Perfusion Cultures

yessotoxin concentration	semicontinuous	perfusion
$[YTX]_{supernatant}$ (ng·mL^{-1})	28.1	37.6
$[YTX]_{cell}$ (pg·cell^{-1})	4	3.57
$[YTX]_{total\ max}$ (ng·mL^{-1})	316	677

authors found an increase in production of C2 toxin, a molecule that contains nitrogen, with the nitrate concentration and inhibition with high phosphate concentration. Nitrate is important for cell growth and aminoacid production, and phosphate is needed for cell division. YTX composition does not contain nitrogen or phosphorus (see Figure 1), so the availability of these elements has no probable direct impact on their production. As can be seen in Table 3, the yield of nutrients in toxins for phosphate is higher than for nitrate in both experiments. These results are in agreement with the biomass nutrient yield, since YTX contents were found to be constant in both experiments. Thus, the higher the efficiency of the nutrient in biomass production, the higher the YTX yield. The perfusion experiment has been shown to be more efficient in nutrient usage (highest Y_{max}), which is why in this reactor the punctual maximum toxin yields of nutrients are obtained. Taking these two facts into account, the perfusion experiment arises as the best option for YTX production from this strain of *P. reticulatum*, since the yield of nutrients in biomass and, as a consequence, in yessotoxins are higher than the other operation mode.

However, toxin production in dinoflagellates has been attributed to a defense strategy to minimize the competitive disadvantages under nutrient limitation (*38*), with higher toxin content in species with a lower affinity to nutrients. If this were the case for *P. reticulatum* the toxin cell content might have changed with changes in nutrients during culture, but this was not observed.

It is difficult to compare the values obtained in the present work with those in the literature, since YTXs in *P. reticulatium* have been found recently (*39*) and very few studies have been carried out with this dinoflagellate. The YTX concentrations obtained were extraordinarily higher than those previously reported: 100 ng·mL^{-1} (*40*) and 57 ng·mL^{-1} (*41*). However, cell YTX content was similar to or lower than the values in the literature: 5.5 pg·cell^{-1} (*42*), 6 pg·cell^{-1} (*23*) and 26 pg·cell^{-1} (*41*). The maximum productivity of YTXs (Δ[toxin]/Δt) took place between days 11 and 20 with a value of 25 ng·mL^{-1}·d^{-1}, which is over 5 times higher than that obtained by Paz et al. (*17*, *40*) ($\sim$5 ng·mL^{-1}·d^{-1}). The toxin content in cells has also been reported to vary notably among strains of the same species (*23*). The discrepancies observed in bibliographic data are therefore to be expected.

Pigments. Figure 9a and b shows the concentration of the main pigments in the perfusion culture and in the cells of *P. reticulatum*. This dinoflagellate possesses two main pigments, peridinin and chlorophyll *a*, and low amounts of dianoxantin. The chlorophyll *a* content in the cells was constant until day 36 (Figure 9b), followed by a sharp increase and a stable interval during the late stationary phase. Peridinin decreased at first and then slightly accumulated until day 33, when the stationary phase started, i.e., when cell concentration was very high, which produced a sharp increase until the end of the culture (Figure 9a). This evolution is expected, since when photoautotrophic cells are highly photolimited, i.e., stationary phase, they may synthesize more light receptors for a higher absorption (*43*). A similar pattern has been observed (*21*) with *Prorocentrum lima* but with light-dark cycle cultures, and an increase in chlorophyll

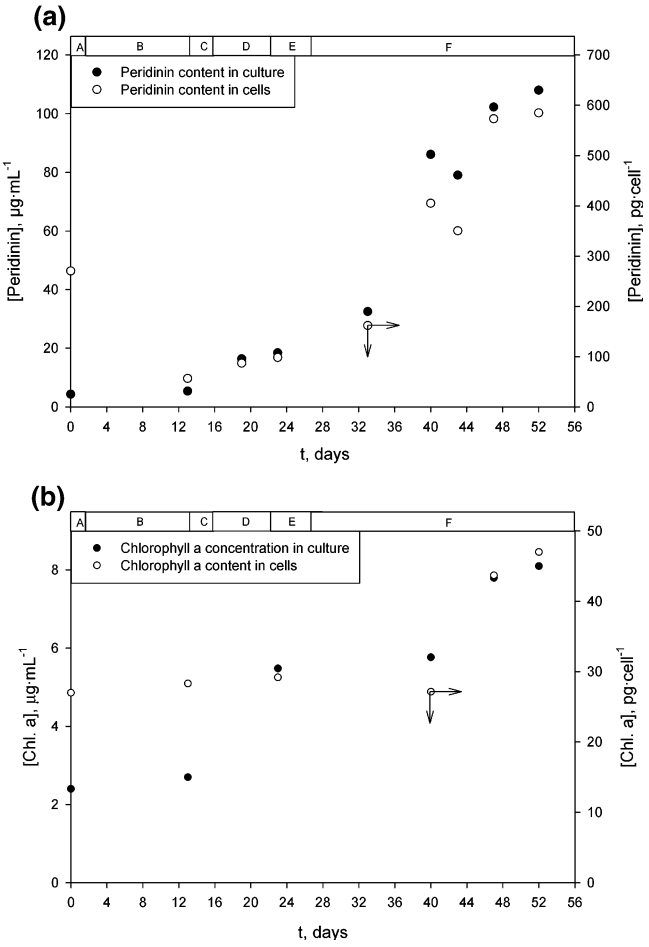

Figure 9. Evolution of pigment concentration in the culture and in the cells for the experiment with the spin-filter: (a) peridinin; (b) chlorophyll *a*.

production has been observed during the night (*44*). However, the trend observed in our cultures cannot be easily explained, as it may be the consequence of various processes due to the complex nature of these microorganisms. The following three processes may therefore be responsible for the pigment concentrations:

(1) Dinoflagellates are species with a very strong circadian cycle controlled by the light cycle. In our cultures irradiance and agitation were continuously applied to the culture, which constituted a considerable change in their natural light cycle with respect to static cultures. This change might have provoked a change in their pigment production, which only increased when the cells were in a very light-limited situation.

(2) The inverse relationship between the pigment and nitrate contents in the medium, as previously reported (*45*). Thus, when the nitrate content increased over the first 40 days, chlorophyll *a* content remained constant despite the reduction in light availability, whereas when the nitrate content decreased and the cell concentration was high (see Figures 7a and 6), the pigment content increased sharply.

(3) Cell growth in this culture was only limited by light, and consequently there was an increase in light-harvesting pigments when the cell number was high, since the light absorption is severe in dense cultures (*46*). Therefore, the pigment content of the cells increased sharply at the time when the stationary phase started on day 36. At the highest cell concentration achieved, the chlorophyll *a* content in the culture was 8.1 $\mu g \cdot mL^{-1}$, which is lower than or similar to those in the literature, depending on the species: 42.2×10^{-3} $\mu g \cdot mL^{-1}$ (*47*), 5.5 $\mu g \cdot mL^{-1}$ with *A. tamarense* (*36*), 49.6×10^{-3} $\mu g \cdot mL^{-1}$ with *P. minimum* (*33*); 0.9 $\mu g \cdot mL^{-1}$ with *P. lima* (*21*), for cell densities 1 order of magnitude lower than those reached in this culture.

The behavior until the stationary growth phase is similar to that previously described for *A. tamarense* (*36*). However, in those results the chlorophyll *a* cell content started to decline slowly after the cells entered the stationary growth phase at the end of the experiment. Their culture entered the stationary growth phase when the phosphate in the medium was depleted. Since our culture growth was not limited by this nutrient, the pigment evolution cannot be the same.

The relationship between peridinin and chlorophyll *a* in the light-harvesting protein complex has been found constant for certain species (*48, 49*). However, the relationship between these two pigments in our culture increased from 2 to 15 g peridinin/g Chl.a from the beginning to the end of the culture. Photosynthetic microorganisms can adapt to different light situations by changing the number of photosynthetic units (PSU) (*50*) or by changing the size of PSU in the chloroplasts (*46, 51*). These two mechanisms might explain the variation in the cell peridinin/chl. a ratio with culture age. A seasonal variation in pigment proportions has also been previously observed in natural cultures of *Peridinium gatunense* (*51*).

Conclusions

In view of the results presented in this work the following conclusions can be drawn:

(1) L1 medium, which is traditionally used for dinoflagellate culture, is clearly deficient in the two main inorganic nutrients, nitrate and phosphate for the strain cultured, since by keeping the concentrations at higher levels with the two feeding strategies, i.e., semicontinuous and perfusion cultures, the highest concentrations ever referenced were obtained.

(2) The great uptake shortly after the addition of nutrients, despite the high concentrations in the culture, lead us to conclude that the cellular metabolism of this strain is very complex and further research is required in order to clarify cellular behavior.

(3) *Protoceratium reticulatum* may be massively cultivated at lab scale in traditional transparent bioreactors. These preliminary results open a door to the production of YTXs and probably other toxins from fragile dinoflagellates on a medium to large scale, since this technology could be easily adapted to other strains.

(4) YTX concentration was proportional to cell concentration, and therefore mass cultures are needed in order to obtain large amounts of these toxins. The culture strategies presented in this work may help to achieve this objective.

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