

Macronutrients requirements of the dinoflagellate *Protoceratium reticulatum*

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ABSTRACT

The basal L1 medium was found to be unsatisfactory for culturing the red tide dinoflagellate *Protoceratium reticulatum* at a high growth rate and biomass yield. The L1 medium enhanced with phosphate to a total concentration of 217 μM supported the highest attainable growth rate and biomass yield. Once the phosphate concentration exceeded $6 \times \text{L1}$, phosphate inhibited the dinoflagellate growth and negatively affected cell viability. At the optimal phosphate concentration of 217 μM , an increase in nitrate concentration over the range of 882–8824 μM , did not affect cell growth and yield. Nitrate did not inhibit growth at any of the concentrations used. Clearly, the basal nitrate level in L1 is sufficient for effectively culturing *P. reticulatum*. At the ranges of phosphate and nitrate concentrations tested, cell volume was not sensitive to the concentration of nutrients but the concentration of phosphate affected both the specific cell number and cell volume growth rates. Elevated levels of nutrients supported their intracellular accumulation. Cell-specific production of yessotoxin was not influenced by concentration of phosphate in the culture medium, but elevated ($>1764 \mu\text{M}$) nitrate concentration did enhance the yessotoxin level. Phosphate concentration that maximized biomass yield also maximized volumetric production of yessotoxin in the culture broth.

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1. Introduction

Blooms of toxic dinoflagellates are associated with fish kills, poisonings of marine wildlife, and seafood poisonings of humans (García Camacho et al., 2007b). Dinoflagellates are very shear-sensitive micro-organisms (García Camacho et al., 2007a) that produce various toxins (García Camacho et al., 2007b). Some of these toxins are of potential interest in human medicine and quantities of pure toxins are also needed as analytical standards that are used in identifying and quantifying toxins in natural samples. Production of dinoflagellate toxins for various purposes requires an understanding of the nutritional requirements for dinoflagellate growth and toxin production (Kobayashi and Kubota, 2007; García Camacho et al., 2007b). The dinoflagellate *Protoceratium reticulatum*, also known as *Gonyaulax grindleyi*, is a producer of yessotoxins (YTXs). *P. reticulatum* is widespread in the world's oceans (Ciminiello et al., 2003; Satake et al., 1997, 1999; Paz et al., 2007) and is of particular concern in shellfish aquaculture (Guerrini et al., 2007). YTXs are disulfated polyether compounds

that are potent cytotoxins. Yessotoxins are further reviewed by Bowden (2006).

Environmental conditions are believed to be important for toxin expression in dinoflagellates and other microalgae. For example, production of paralytic shellfish poisoning (PSP) toxins by *Alexandrium* spp. appears to be higher at low concentrations of phosphorous (Anderson et al., 1990; Beani et al., 2000) and low salinity values (Hwang and Lu, 2000). The effect of environmental factors on the total amount of toxins produced has been found to be highly dependent on algal species (Guerrini et al., 2007).

This work reports on the production of yessotoxins by *P. reticulatum* as a potential commercial producer for this compound for use in research. Only a few studies have focused on YTX production in this dinoflagellate. Seamer (2001) investigated the effects of nutrients, light, salinity and temperature on YTX production. Mitrovic et al. (2004) evaluated the effects of selenium, iron and cobalt supplementation on growth and YTX production. Paz et al. (2006) examined the influence of temperature, irradiance and salinity on a *P. reticulatum* strain from Spain. Guerrini et al. (2007) reported on the effects of nitrate and phosphate supplementation on growth and YTX production. Toxin production in toxic dinoflagellates has been found to be elevated under low phosphate conditions (Frangópulos et al., 2004; Guerrini et al.,

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2007), although most studies agree that nitrogen or phosphorus limitation reduces growth of dinoflagellates (Gallardo Rodríguez et al., 2007; Shi et al., 2005; Siu et al., 1997; Wang and Hsieh, 2002).

2. Materials and methods

2.1. Species and culture medium

P. reticulatum strain GG1AM kindly donated by the Centro Oceanográfico de Vigo, Spain, was used. This strain had been isolated from the Mediterranean Sea and was already adapted to grow in the L1 medium (Guillard and Hargraves, 1993) that was used in all experiments. The L1 medium was prepared in Mediterranean Sea water. The pH was adjusted to 8.7 with 3 M HCl or NaOH. The medium was filter sterilized (0.22 μm Millipore filter; Millipore Corporation, Billerica, MA, USA). The salinity was approximately 35‰. The typical composition of the Mediterranean Sea water has been published (Contreras et al., 1998). The inocula were maintained in thermostated chambers at 17 °C in 2 L static Erlenmeyer flasks. A 12:12 h light/dark cycle was used with an average irradiance on the surface of culture of 50 $\mu\text{E m}^{-2} \text{s}^{-1}$. Cool white fluorescent lamps (30 W) were used for lighting. Every 7 days 40% of the inoculum by volume was replaced with fresh L1 medium.

2.2. Growth experiments

Two series of experiments were carried out, as follows:

- (1) In the first series, the initial phosphate concentration in the medium was varied in separate experiments, starting from 36.23 μM , the basal phosphate concentration of the L1 medium. The phosphate concentrations in separate experiments were 1 \times L1 (i.e. 36.2 μM), 2 \times L1, 4 \times L1, 6 \times L1, 8 \times L1 and 10 \times L1 (i.e. 362.3 μM). The initial nitrate concentration was always 882.42 μM , i.e. the basal level in the L1 medium.
- (2) In the second series of experiments, the initial nitrate concentration in the medium increased in steps in separate cultures from that of the basal L1 medium (i.e. 882.42 μM) by a factor of 10. In this series, the phosphate concentration was held constant at the level that had been identified in the series 1 experiments to give the best biomass production results.

Experiments were carried out in static fed-batch cultures in 100 mL Erlenmeyer flasks filled with 50 mL of freshly inoculated *P. reticulatum* broth. Initial cell concentration was about 10,000 cells mL^{-1} in all cases. A culture in mid-exponential growth was used for inoculation. For inoculation, the cells were recovered by centrifugation (75 \times g) and resuspended in fresh medium of appropriate composition that depended on the specific experiment. Flasks were arranged in rows and were illuminated on one side by two white fluorescent lights. All flasks received the same irradiance level of 50 $\mu\text{E m}^{-2} \text{s}^{-1}$ on the illuminated side. The same inoculum was used for all flasks in a given series. All assays of macronutrients were carried out in duplicate. At the end of the culture run, a 15-mL sample was taken from each flask for determination of yessotoxins.

Samples for measuring nitrate, phosphate and the cell concentration were taken at the same time (9:00 a.m.) each day after gentle manual mixing of the flasks. Samples were analyzed immediately. After determining the concentrations of macronutrients, highly concentrated aliquots of nitrate and phosphate were added to the cultures (always before 10:30 a.m.) in order to replenish the macronutrients that had been consumed by the cells during the previous day.

For measuring cell growth, a 1-mL sample was fixed with Lugol's solution (Paz et al., 2004) by mixing a 2:100 (v/v) ratio of Lugol's solution and the sample (Gallardo Rodríguez et al., 2007). Cells were counted immediately under a light microscope using a Sedgewick-Rafter chamber.

2.3. Determination of phosphate and nitrate concentrations

Phosphorous species were measured as PO_4^{3-} using the well-known method of the American Public Health Association (APHA, 1995). Nitrate was quantified by HPLC using a UV detector (Gallardo Rodríguez et al., 2007; Moya, 1996).

2.4. Yessotoxins

Yessotoxins were measured separately in the biomass and in culture supernatant following the method of Paz et al. (2004). An HPLC system (Shimadzu AV10; Shimadzu Corporation, Kyoto, Japan) with a fluorescence detector (Shimadzu RF-10AX) and an autoinjector (Shimadzu SIL-10ADVP) was used.

2.5. Cell volume

Mean cell diameter was measured by forward light scatter method using a Coulter Epics[®] XL-MCL (Beckman Coulter, Inc., Fullerton, CA, USA) flow cytometer. The cytometer was calibrated by measuring the forward scatter of light caused by different-sized latex beads (5, 10, 15, 20, 25 and 30 μm). Cell volume was calculated as $\pi D_e^3/6$ where D_e was the diameter of a sphere that at an equivalent concentration as the cells in suspension, produced the same forward scatter as the suspension of cells (Hillebrand et al., 1999; Paz et al., 2007).

2.6. Cell viability

To quantify the fraction of viable cells, 100 μL of fluorescein diacetate (1 $\mu\text{g mL}^{-1}$) was added to 1 mL of cell suspension. Esterases in viable cells convert fluorescein diacetate to a fluorescent compound that emits at 505–545 nm. Fraction of viable population was calculated as the ratio of the number of fluorescent events detected at the above wavelength and the total cell count measured by the cytometer.

2.7. Free internal nutrients

To quantify free internal nitrate and phosphate 1 mL of culture was centrifuged (75 \times g) and the supernatant was discarded. Afterwards, the cell pellet was disaggregated in 1 mL of demi-water and subjected to sonication for 10 min for cell break-up. The suspension was then centrifuged (1000 \times g) and nutrients were measured in the clear supernatant as described above.

2.8. Kinetic parameters

The specific cell growth rate μ_{cell} (day^{-1}) was calculated using the following equation:

$$\mu_{\text{cell}} = \frac{\ln N_{i+1} - \ln N_i}{t_{i+1} - t_i}, \quad (1)$$

where N_{i+1} and N_i are the cell concentrations (cells mL^{-1}) at times t_{i+1} and t_i (day), respectively. The specific cell volume growth rate μ_{vol} (day^{-1}) was calculated using the following equation:

$$\mu_{\text{vol}} = \frac{\ln V_{i+1} - \ln V_i}{t_{i+1} - t_i}, \quad (2)$$

where V_{i+1} and V_i are the total volume of cells in a given volume of broth (i.e. NV_c , pL mL⁻¹), where N is the number of cells per microliter of broth and V_c is the average volume of a cell in picoliter at times t_{i+1} and t_i (day), respectively.

The cell-specific uptake rate of nutrient S (i.e. ρ_S , $\mu\text{mol h}^{-1} \text{cell}^{-1}$) was calculated with the equation:

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

where S_{i+1} and S_i are the nutrient concentrations (mol L⁻¹) at times t_{i+1} and t_i (day), respectively, and \bar{N} is the average cell concentration over the time interval $t_{i+1} - t_i$.

The nutrient quota per cell (Q_S , mol cell⁻¹) was calculated as follows:

$$Q_S = \frac{S_i - S_{i+1}}{\bar{N}}. \quad (4)$$

From Eqs. (3) and (4),

$$\rho_S = \frac{Q_S}{24(t_{i+1} - t_i)}. \quad (5)$$

3. Results and discussion

3.1. Cell growth and nutrient uptake

Although the L1 medium has been most commonly used in culturing dinoflagellates (Guillard and Hargraves, 1993), it has been found unsatisfactory for supporting a high concentration of biomass (Gallardo Rodríguez et al., 2007). This detailed study of macronutrients requirements of *P. reticulatum* was carried out in order to identify media that better support high cell densities than the conventional L1 medium. The macronutrients that are most important to dinoflagellate growth are nitrate and phosphate. For example, phosphate and nitrate are recognized as being important in development of algal blooms (Paerl, 1998; Sakka et al., 1999; Shi et al., 2005), but most studies of uptake of these nutrients by dinoflagellates have been carried out at relatively low concentrations of nutrients. This is because in nature a relatively slight elevation in concentration of nutrients is sufficient for the development of algal blooms. For example, a nitrogen concentration of 1.94 $\mu\text{mol L}^{-1}$ has been found to induce algal blooms (Fan et al., 2003). As a consequence of limited availability of nutrients, algal blooms typically sustain only a low concentration of biomass (Koike et al., 2006). In bioreactor culture, the aim is generally to maximize growth rate and biomass yield and, therefore, more nutrients need to be provided than would be found typically in natural blooms. This study, therefore, examined various formulations of L1 medium with enhanced levels of phosphate and nitrate, in attempts to enhance productivity of *P. reticulatum* cultures.

As shown in Fig. 1, cell growth rate and final yield were relatively low in low-phosphate media, i.e. media 1× L1 and 2× L1. Growth rate and yield increased progressively as the phosphate concentration increased to 4× L1 and 6× L1; however, a further increase in phosphate level to 8× L1 and 10× L1 actually reduced the cell growth rate and final yield relative to the medium 6× L1 that performed the best (Fig. 1). Inhibitory effects of excess phosphate on dinoflagellates have been referenced (Dyhrman and Palenik, 1999) but the causes have not yet been identified. Most earlier studies were limited to relatively low concentrations of phosphorous sources. Changes in phosphate concentration did not influence the duration of the 1-day lag phase (Fig. 1).

Although phosphate concentration influenced cell growth rate and yield it had no systematic observable effect on cell volume

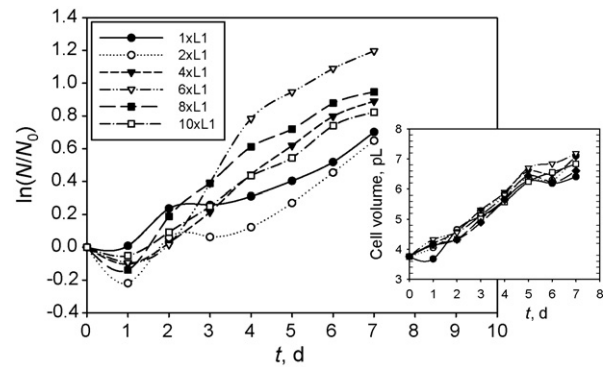


Fig. 1. Normalized cell concentration and cell volume (inset) versus time in the phosphate series.

(Fig. 1, inset). In all media, the cell volume increased from around 4 pL cell⁻¹ to around 7 pL cell⁻¹ during 7 days of culture. Significantly, the rate of increase in cell volume slowed in all media on day 5, as the cells apparently attained the upper limit on size (Fig. 1, inset).

As shown in Table 1, the cell viability at various stages of growth (Fig. 1) was generally high at >90% in most cases in media with phosphate levels of 1–6× L1. Other than at inoculation, the media with higher phosphate levels (i.e. media 8× L1 and 10× L1) always produced a cell population with a significantly lower viability than the other media (Table 1); nevertheless, the viability was generally >65%.

Ratios of the specific cell volume growth rate and the specific cell number growth rate (i.e. μ_{vol}/μ_{cell}) at various stages of growth in various media are shown in Table 1. Irrespective of the medium and the stage of growth, μ_{vol}/μ_{cell} was substantially greater than 1.0; thus, cells increased in volume significantly faster with time compared to the increase in cell number.

Cell-specific consumption rate of phosphate relative to the blank is shown in Fig. 2 for various initial concentrations of phosphate in the medium (in both series of experiments, the cultures with the lowest provided concentration of nutrients within a series were taken as 'blanks'). Compared at the same time since inoculation in different media, the consumption rate of phosphate increased with increasing initial concentration of phosphate in the medium. At any given initial concentration of phosphate, the relative specific consumption rate increased with time from day 1 to day 3, i.e. during the early exponential growth phase. Cell-specific phosphate uptake rate peaked on day 6 and then declined substantially on day 7 (Fig. 2). As mentioned previously, a clear peak is seen in the cell-specific growth and volume rates at a phosphate concentration of around 220 μM (Fig. 2).

Cell quota of a nutrient is an indicator of average uptake of that nutrient by the cells. Data on cell quotas (pmol cell⁻¹) of N and P at various stages of growth in the six media in both series runs are shown in Tables 2 and 3. In general at all stages of culture, the cell quota for N was always greater than the cell quota for P, i.e. much more N was consumed compared to P. This is expected in normal metabolism of dinoflagellates and other microalgae, as on a mole basis a microalgal cell contains roughly 10-times as much N as P (Chisti, 2007). Phosphate quotas decreased with time in both series but no systematic variation with time and P-concentration could be identified for the N quota. It is interesting to note that when nitrate concentration was higher than 882 μM (1× L1), after the lag phase, there was a release to the medium of this nutrient (negative cell quotas). This has been previously observed in *P. reticulatum* (Gallardo Rodríguez et al., 2007).

Table 1
Cell viability and μ_{vol}/μ_{cell} in the phosphate series runs

| t (day) | Phosphate concentration | | | | | | | | | | | |
|---------|-------------------------|------------------------|--------|------------------------|--------|------------------------|--------|------------------------|--------|------------------------|---------|------------------------|
| | 1 × L1 | | 2 × L1 | | 4 × L1 | | 6 × L1 | | 8 × L1 | | 10 × L1 | |
| | V (%) | μ_{vol}/μ_{cell} | V (%) | μ_{vol}/μ_{cell} | V (%) | μ_{vol}/μ_{cell} | V (%) | μ_{vol}/μ_{cell} | V (%) | μ_{vol}/μ_{cell} | V (%) | μ_{vol}/μ_{cell} |
| 0 | 99 | | 99 | | 99 | | 99 | | 99 | | 99 | |
| 1 | 94 | 2.30 | 94 | 1.93 | 90 | 1.74 | 88 | 1.36 | 88 | 1.37 | 86 | 1.14 |
| 2 | 88 | | 97 | | 92 | | 79 | | 79 | | 78 | |
| 3 | 97 | | 93 | | 95 | | 87 | | 84 | | 72 | |
| 4 | 97 | 1.26 | 93 | 1.25 | 95 | 1.21 | 91 | 1.31 | 86 | 1.39 | 70 | 1.30 |
| 5 | 97 | | 93 | | 94 | | 95 | | 86 | | 68 | |
| 6 | 91 | | 93 | | 97 | | 94 | | 81 | | 72 | |
| 7 | 98 | | 98 | | 95 | 1.63 | 91 | 1.28 | 79 | 2.46 | 66 | 1.47 |

V, viability; μ_{vol} , specific cell volume growth rate; μ_{cell} , specific cell number growth rate.

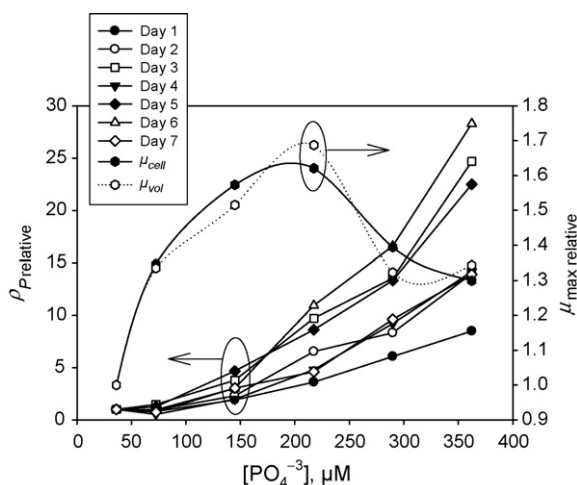


Fig. 2. Specific rate of phosphate consumption relative to blank and specific growth rates versus initial phosphate concentration, at various times during culture.

In general the cell quotas of P declined with time (Tables 2 and 3), as progressively less phosphate per cell was available. Because at 6 × L1 phosphate concentration, the medium already had sufficient P to support a high cell concentration, the high P uptake values in media 8 × L1 and 10 × L1 suggest that more P was being taken up than was necessary for normal metabolism. In presence of excess P, microalgae are known to accumulate it as polyphosphate.

In Fig. 3, the molar ratio of N and P consumed by a certain time are plotted against the initial value of the N:P in the medium. Clearly, the initial value of N:P in the medium affects the relative rates of consumption of these nutrients.

In experimental series II, the concentration of phosphate in the medium was held constant at 217.4 μM that was identified to be

optimal for attaining a high growth rate and final cell yield (see series I experiments), but the concentration of nitrate increased in different batches of the medium. As shown in Fig. 4, the cell growth rate and final yield were barely influenced by changes in nitrate concentration. This is further revealed in Fig. 5. Clearly, the basal L1 medium has plenty of nitrate for supporting the growth yield and rate seen here.

A comparison of Figs. 1 and 4 clearly reinforces that phosphate in the basal L1 medium is insufficient, but nitrate is. Similarly, changes in nitrate concentration did not influence the rate of increase in cell volume (Fig. 4, inset). In this case there was a 2-day lag phase but, again, cell volume increased from day 0 (Fig. 4, inset). Although nitrate level did not influence growth rate and final cell yield, it did affect the length of the lag phase. The lag phase consistently was 2-days (Fig. 4) instead of 1-day that was observed in the phosphate series of runs (Fig. 1). Cells obviously required some time to adapt to changes in concentration of nitrate, but otherwise were not affected. Nitrate concentration did not have any systematic adverse effect on cell viability which was mostly >90% (Table 4).

Although both N and P are essential nutrients for microalgae, a substantially greater molar quantity of N is always required compared with the quantity of P, but too much nitrogen can have a negative effect. For example, a N:P ratio of 8–24 has promoted cell growth and improved biomass yield of the dinoflagellate *Alexandrium tamarense*, but growth and yield were reduced once the N:P ratio exceeded about 73 (Shi et al., 2005). Within limits, an excess of P can be tolerated. Consumption of P without production of biomass has been reported (Sakka et al., 1999) and is related to intracellular accumulation of P, as pointed out earlier. A phytoplankton culture medium is considered balanced when the N:P molar ratio is close to 16 (i.e. the Redfield ratio) (Purina et al., 2004); however, in media that are rich in all nutrients, the specific value of the N:P ratio has little bearing on growth rate and final yield, as seen in this study.

Table 2
Cell quotas (pmol cell^{-1}) of N and P in phosphate series runs

| t (day) | Phosphate concentration | | | | | | | | | | | |
|---------|-------------------------|------|--------|------|--------|------|--------|------|--------|------|---------|-------|
| | 1 × L1 | | 2 × L1 | | 4 × L1 | | 6 × L1 | | 8 × L1 | | 10 × L1 | |
| | N | P | N | P | N | P | N | P | N | P | N | P |
| 1 | 8.96 | 1.30 | 7.72 | 1.06 | 10.70 | 2.47 | 7.50 | 4.68 | 8.69 | 7.86 | 8.57 | 11.02 |
| 2 | 12.40 | 0.72 | 10.17 | 0.68 | 4.21 | 1.65 | 7.07 | 4.69 | 3.80 | 5.97 | 9.74 | 10.03 |
| 3 | 8.79 | 0.36 | 4.56 | 0.53 | 8.63 | 1.35 | 8.48 | 3.48 | 9.22 | 4.85 | 5.53 | 8.83 |
| 4 | 9.18 | 0.80 | 12.51 | 0.42 | 11.04 | 1.58 | 8.19 | 3.83 | 8.48 | 7.35 | 8.15 | 11.31 |
| 5 | 10.91 | 0.62 | 11.10 | 0.78 | 9.10 | 2.88 | 7.60 | 5.31 | 6.95 | 8.22 | 8.10 | 13.86 |
| 6 | 12.56 | 0.35 | 9.55 | 0.34 | 6.76 | 2.28 | 6.99 | 3.87 | 5.17 | 5.86 | 8.08 | 10.00 |
| 7 | 3.73 | 0.35 | 3.45 | 0.26 | 4.44 | 1.07 | 3.22 | 1.61 | 5.97 | 3.37 | 4.73 | 4.88 |

Table 3
Cell quotas (pmol cell^{-1}) of N and P in nitrate series runs

| t (day) | Nitrate concentration | | | | | | | | | | | |
|---------|-----------------------|-------|--------|-------|--------|------|--------|------|--------|------|---------|-------|
| | 1 × L1 | | 2 × L1 | | 4 × L1 | | 6 × L1 | | 8 × L1 | | 10 × L1 | |
| | N | P | N | P | N | P | N | P | N | P | N | P |
| 1 | 5.18 | 11.31 | 8.25 | 10.48 | 13.60 | 9.89 | 28.46 | 9.88 | -12.18 | 9.45 | 57.68 | 10.27 |
| 2 | 6.88 | 10.52 | 9.68 | 9.07 | 18.83 | 9.10 | 39.71 | 8.78 | 32.28 | 8.27 | 25.14 | 9.49 |
| 3 | 3.16 | 8.97 | 8.01 | 7.68 | -1.99 | 8.64 | -1.78 | 8.45 | -18.50 | 8.28 | -12.88 | 7.83 |
| 4 | 2.67 | 8.21 | -0.78 | 7.90 | -3.21 | 7.92 | -8.86 | 7.80 | -9.62 | 7.39 | -0.88 | 6.86 |
| 5 | 5.86 | 6.64 | 11.13 | 5.53 | 11.70 | 5.72 | 18.46 | 5.80 | 9.09 | 5.24 | 11.66 | 5.15 |
| 6 | 8.20 | 5.08 | 6.43 | 4.74 | 10.84 | 4.47 | 16.79 | 4.56 | -1.04 | 4.72 | 28.42 | 4.24 |
| 7 | 2.94 | 1.97 | -4.94 | 1.86 | -18.74 | 2.05 | -5.98 | 2.24 | -36.45 | 1.92 | -16.08 | 2.01 |

In keeping with the present work, studies with *A. tamarense* suggest that the L1 medium is nitrogen sufficient (Leong and Taguchi, 2004), but reducing nitrogen concentration negatively impacts growth once the nitrogen level falls below $100 \mu\text{M}$ (Gallardo Rodríguez et al., 2007; Leong and Taguchi, 2004). With *A. tamarense* a negative effect on growth, of excess nitrogen has been reported for nitrogen level of $4 \times \text{L1}$ (Shi et al., 2005), but with *P. reticulatum* we did not see any growth inhibitory effects at nitrate levels as high as $10 \times \text{L1}$. Our observation with *P. reticulatum* is consistent with similar reports for other dinoflagellates (Hoe Chang and McClean, 1997; Siu et al., 1997).

Irrespective of the media used, similar patterns of increase in cell volume were observed with time. This suggests that increase in volume is an intrinsic characteristic of growth of *P. reticulatum* and is not evidently influenced by nitrate and phosphate over the ranges of concentrations that were examined (Figs. 1 and 4). Dinoflagellates are not generally known to accumulate nitrogen within cells under conditions of surfeit nitrogen in the medium (Flynn and Flynn, 1998; Leong and Taguchi, 2004; Lomas and Glibert, 1999, 2000), but intracellular accumulation of phosphate is known (Touzet et al., 2007) and has the potential to increase the volume of the cell because of the presence of polyphosphate granules. To determine which variables were responsible of the cell volume increase observed in Figs. 1 and 4 a multiple regression of volume data of the two series using Statgraphics v.5.1 was carried out. The analysis' results showed that only time, free internal phosphate concentration (pmol pL^{-1}) and free phosphate content in the cells (pmol cell^{-1}) had a statistically significant effect (p -values obtained < 0.0001) on volume. Therefore, the observed increase in cell volume cannot be exclusively ascribed to cell age but also to internal phosphate content.

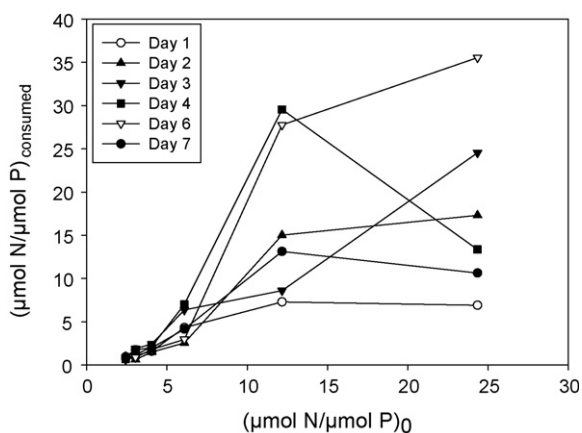


Fig. 3. Ratio of nitrate to phosphate consumed versus initial molar ratio of N:P in the phosphate series.

In Fig. 6 are represented internal nutrient content of the cells (pmol cell^{-1}) and nutrient concentration considering the whole cell volume (pmol pL^{-1}). There is a direct relationship between nutrient concentration within the cells and nutrient concentration in the cells for the two nutrients; this indicates that, despite the volume increase, nutrients are actively transported against the driving force to the cell's interior. This active transport will probably consume a high amount of energy. In that process to

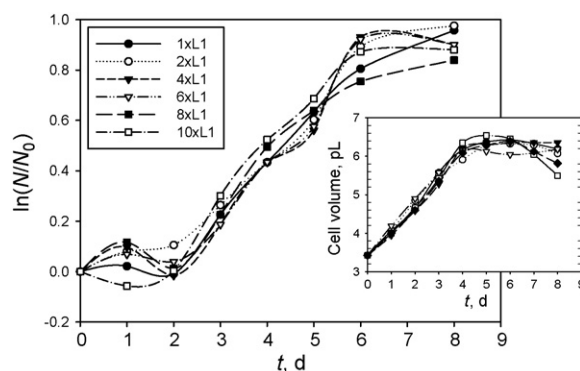


Fig. 4. Normalized cell concentration and cell volume (inset) versus time in the nitrate series of runs.

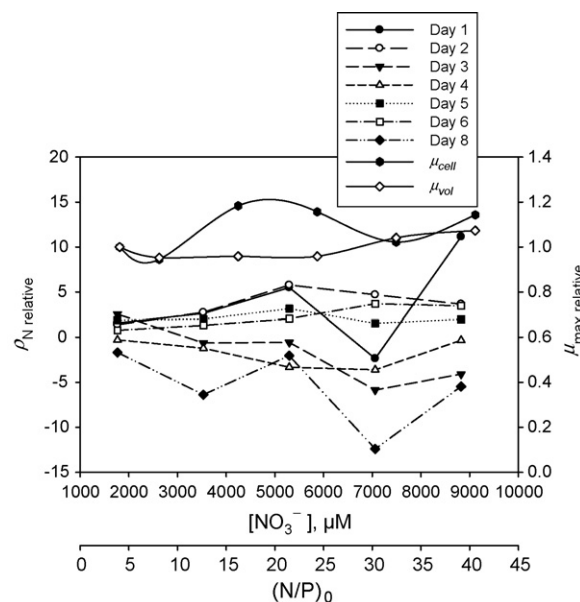


Fig. 5. Relative nitrate cell consumption rate and relative maximum-specific growth rates versus nitrate concentration.

Table 4
Cell viability and μ_{vol}/μ_{cell} in the second series runs

| t (day) | Nitrate concentration | | | | | | | | | | | |
|---------|-----------------------|------------------------|-------|------------------------|-------|------------------------|-------|------------------------|-------|------------------------|--------|------------------------|
| | 1× L1 | | 2× L1 | | 4× L1 | | 6× L1 | | 8× L1 | | 10× L1 | |
| | V (%) | μ_{vol}/μ_{cell} | V (%) | μ_{vol}/μ_{cell} | V (%) | μ_{vol}/μ_{cell} | V (%) | μ_{vol}/μ_{cell} | V (%) | μ_{vol}/μ_{cell} | V (%) | μ_{vol}/μ_{cell} |
| 0 | 94 | | 94 | | 94 | | 94 | | 94 | | 94 | |
| 1 | 92 | | 90 | | 88 | | 92 | | 87 | | 88 | |
| 2 | 89 | 1.37 | 88 | 1.39 | 82 | 1.11 | 88 | 1.10 | 81 | 1.40 | 83 | 1.29 |
| 3 | 92 | | 91 | | 87 | | 91 | | 88 | | 88 | |
| 4 | 95 | | 94 | | 92 | | 93 | | 94 | | 93 | |
| 5 | 94 | 0.80 | 94 | | 94 | | 95 | | 96 | | 95 | |
| 6 | 93 | | 94 | 0.52 | 97 | 1.00 | 97 | 0.04 | 97 | -0.03 | 96 | -1.84 |
| 7 | 94 | | 95 | | 95 | | 95 | | 96 | | 95 | |
| 8 | 94 | | 96 | | 93 | | 94 | | 95 | | 94 | |

V, viability; μ_{vol} , specific cell volume growth rate; μ_{cell} , specific cell number growth rate.

attain similar internal amounts of nitrate than of phosphate, nitrate concentration in the medium has to be more than 20 times higher (8824 $\mu\text{M}/360 \mu\text{M}$), but it is clear that nitrate was actively accumulated within the cells of *P. reticulatum*, since its concentration (up to 4.21 M at 10× L1 in the nitrate series) is much higher than the concentration in the medium ($8.82 \times 10^{-3} \text{ M}$).

Increase in size of cells means that the surface to volume ratio of the cell declines with age. In some cases, this would imply a reduced rate of uptake of nutrients per unit volume of the cell, as the cell ages. Changes in cell size have been reported to be important in influencing nutrient uptake and, therefore, cell-specific growth rate (Lomas and Glibert, 2000). The observed increase in cell volume was of the same order of magnitude as reported by others (Guerrini et al., 2007), despite being measured with different techniques. In some dinoflagellates, increase in cell volume with age is known to be influenced by the composition of the culture medium (Guerrini et al., 2007).

3.2. Yessotoxin production

Although the production of some toxins by dinoflagellates has been shown to depend on the nature of the light–dark cycle (Pan et al., 1999; Proctor et al., 1975; Siu et al., 1997) and magnitude of irradiance (Paz et al., 2006), these aspects were not investigated, as they are already known. For a fixed light–dark cycle and irradiance

regimen, the effects of the concentrations of phosphate and nitrate on yessotoxin production are shown in Table 5.

Over the concentration range of 36–362 μM , phosphate had no influence on the YTX content per cell and YTX concentration within cells (Table 5) was constant, even though the phosphate concentration affected the specific number growth rate of cells and the final biomass yield (Fig. 1). Over this concentration range, the N:P ratio varied from 2.4 to 24.3. Toxin production by *P. reticulatum* has been reported to be enhanced under severe phosphate limitation (Guerrini et al., 2007), but this situation obviously did not occur in the present study.

During culture, the cell volume of *P. reticulatum* generally increased from about 4 pL to about 7 pL, depending on the stage of growth (Figs. 1 and 4); however, except at the inoculation stage, the average volumetric concentration of YTX within cells was generally within 0.4–0.6 pg pL^{-1} , irrespective of the phosphate concentration (Table 5) and at a constant nitrate level of 882 μM . *P. reticulatum* is known to release an increasing amount of YTX from the cells to the exterior, as the culture ages (Gallardo Rodríguez et al., 2007; Paz et al., 2007) but, since the culture time was very short, this did not occur in the present study and the amount of yessotoxins in the supernatant was always quite low, often below the detection limit of the HPLC system.

In the L1 medium, increasing the concentration of nitrate to 4× L1, 6× L1 and 8× L1, did significantly increase the YTX

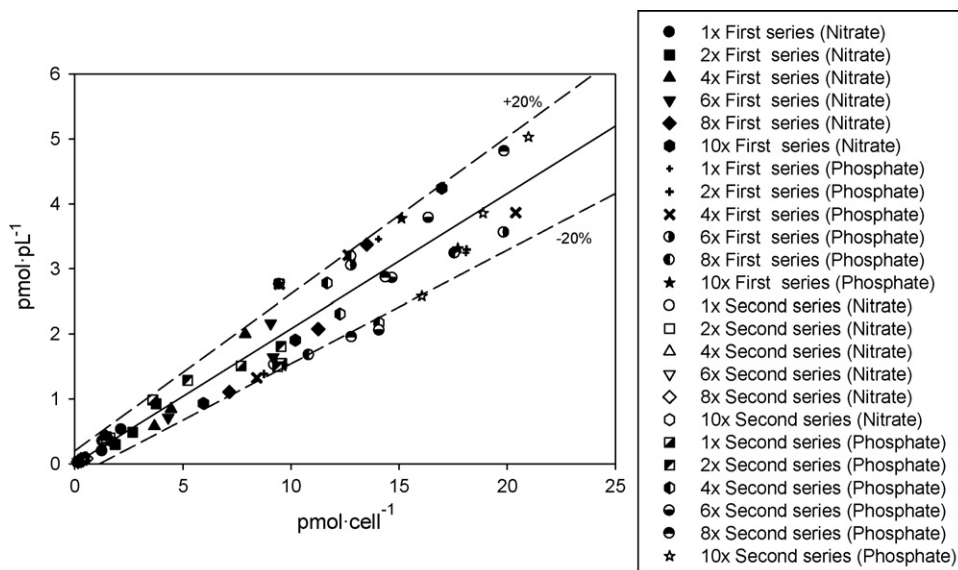


Fig. 6. Relationship between nutrient content and nutrient concentration in the cells in both series for both nutrients.

Table 5
Yessotoxin concentration in cells in various media

| Nitrate (μM) | Phosphate (μM) | N:P | Yessotoxin in cells (pg cell^{-1}) | Yessotoxin concentration in cells (pg pL^{-1}) |
|---------------------------|-----------------------------|------|---|---|
| Inoculum | | 24.3 | 3.2 ± 0.2 | 0.8 |
| 882 | 36.2 | 24.3 | 3.8 ± 0.5 | 0.6 |
| 882 | 72.5 | 12.2 | 3.1 ± 0.7 | 0.4 |
| 882 | 144.9 | 6.1 | 3.7 ± 0.6 | 0.5 |
| 882 | 217.4 | 4.1 | 3.2 ± 0.3 | 0.5 |
| 882 | 289.8 | 3.0 | 3.7 ± 0.1 | 0.5 |
| 882 | 362.3 | 2.4 | 3.6 ± 0.4 | 0.5 |
| Inoculum | | 24.3 | 3.1 ± 0.2 | 0.9 |
| 882 | 217.4 | 4.1 | 2.8 ± 0.3 | 0.4 |
| 1764 | 217.4 | 8.1 | 3.2 ± 0.4 | 0.5 |
| 3528 | 217.4 | 16.2 | 5.0 ± 0.8 | 0.8 |
| 5292 | 217.4 | 24.3 | 4.6 ± 0.3 | 0.7 |
| 7056 | 217.4 | 32.5 | 4.9 ± 0.6 | 0.9 |
| 8820 | 217.4 | 40.6 | 3.7 ± 0.3 | 0.6 |

concentration per cell (Table 5). Nitrate concentrations outside this range were not beneficial. No clear influence of the N:P ratio on production of YTX could be discerned. YTX molecule does not contain N and P (Ferrari et al., 2004). Therefore, any influence of these nutrients on productivity of YTX can be explained only by their possible effects on the enzymes that are directly or indirectly involved in the production of YTX.

The GG1AM strain used here is apparently a moderate producer of YTX compared to some of the other known strains. Other *P. reticulatum* strains, for example strains VGO758 and VGO764, produce 10-times as much YTX as does the strain GG1AM in a given time (Paz et al., 2007). A New Zealand strain of the dinoflagellate *Gonyaulax spinifera* has been reported to contain YTX at 200 pg cell^{-1} (Rhodes et al., 2006), but the cells of this dinoflagellate are much larger (about $40 \mu\text{m}$ in diameter) than *P. reticulatum*. On a cell volume basis, *G. spinifera* has about 4.2 pg pL^{-1} intracellular YTX, a level that is closer to the values here presented. In comparison with our values, the literature reports a quite broad range of YTX levels in cells: 5.5 pg cell^{-1} (MacKenzie et al., 2002), 6 pg cell^{-1} (Satake et al., 1999), 26 pg cell^{-1} (Aasen et al., 2005), and 4 pg cell^{-1} (Gallardo Rodríguez et al., 2007).

4. Concluding remarks

Because of phosphate deficiency, the basal L1 medium is unsatisfactory for culturing *P. reticulatum* at a high growth rate and biomass yield. The L1 medium supplemented with phosphate to a concentration of $217 \mu\text{M}$ provides optimal biomass growth rate and yield, but higher concentrations of phosphate inhibit cell growth and adversely impact cell viability. The nitrate level in L1 is sufficient for culturing *P. reticulatum* and increasing the nitrogen level to $10\times$ L1 neither improves nor inhibits cell growth. High phosphate and nitrate levels cause their intracellular accumulation. Concentration of yessotoxin in *P. reticulatum* is not influenced by changes in phosphate concentration in the culture medium over the concentration range examined, but conditions that support a high biomass yield also lead to a high total production of yessotoxin in the culture broth. At $217 \mu\text{M}$ phosphate, elevated levels of nitrate ($>1764 \mu\text{M}$) positively influence the intracellular concentration of yessotoxin.

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