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Preliminary studies on the significance of alkaline phosphatase activity in the diatom *Phaeodactylum tricornutum* Bohlin*

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ABSTRACT: Alkaline phosphatase activity (APA) and total cellular phosphorus were investigated in batch culture of the diatom *Phaeodactylum tricornutun* Bohlin. The alga used three phosphomonoesters as P source: p-nitrophenylphosphate (*p*NPP), methyl-umbelliferylphophate (MUP) and ß-glycerophosphate. The enzyme exhibited an apparent negative cooperativity for the hydrolysis of *p*NPP and MUP. Affinity was higher for MUP than for *p*NPP, but maximum velocity was similar. Incubation at phosphate concentration < 50 µM shows no effect on APA, but the enzymatic activity decreased markedly at higher phosphate concentrations. The decrease depended on the time of incubation (4 days), suggesting repression of enzyme synthesis. In cultures supplied with ß-glycerophosphate the enzyme utilised preferentially this substrate, instead of MUP. Phosphatase activity was inversely related with total phosphorus content. The ability to take up phosphate and APA increased markedly with increased phosphorus deficiency. Maximum APA (27 fg MU cell^{-1} h^{-1}) was observed when phosphate uptake was high (V_{max}=6.5 fm P cell^{-1} h^{-1}) and phosphorus content was low (0.33 pg P cell^{-1}). In contrast, a minimum APA (1.4 fg MU cell^{-1} h^{-1}) was associated to low phosphate uptake (less than 1 fm P cell^{-1} h^{-1}) and maximum phosphorus content (9.6 pg P cell^{-1}).

Key words: Alkaline phosphatase activity, phosphate uptake, *Phaeodactylum tricornutum.*

RESUMEN: ESTUDIOS PRELIMINARES SOBRE LA ACTIVIDAD FOSFATASA ALCALINA EN LA DIATOMEA *PHAEODACTYLUM TRICORNUTUM* BOHLIN. – Se ha estudiado la actividad fosfatasa alcalina (APA) y el contenido interno de fósforo en cultivos cerrados en la diatomea *Phaeodactylum tricornutun* Bohlin. El alga usó los tres fosfomonoesteres utilizados como fuente de fósforo: p-nitrofenilfosfato (*p*NPP), metil-umbelliferilfosfato (MUP) and ß-glicerofosfato. Se encontró cooperatividad negativa en la hidrólisis del *p*NPP y el MUP. La afinidad fue mayor para el MUP que para el *p*NPP, sin embargo la velocidad máxima fue similar para ambos sustratos. Incubaciones a concentraciones de fosfato menores de 50 µM no tuvieron efecto alguno sobre la APA, pero la actividad enzimática disminuyó notablemente a mayores concentraciones. La disminución en la actividad estuvo relacionada con el tiempo de incubación (4 días), sugiriendo la represión de la síntesis enzimática. En cultivos suministrados con ß-glicerofosfato, el enzima utilizó preferentemente este sustrato, cuando el ensayo se realizó con MUP. La actividad fosfatasa estuvo inversamente relacionada con el contenido interno de fósforo. La tasa de incorporación de fósforo y la APA se incrementaron marcadamente al aumentar la decificiencia en fósforo. La máxima APA (27 fg MU cell^{-1} h^{-1}) fue calculada cuando la incorporación de fósforo fue elevada (V_{max}=6.5 fm P cell^{-1} h^{-1}) y el contenido en fósforo bajo (0.33 pg P cell^{-1}). Por otra parte, una APA mínima (1.4 fg MU cell^{-1} h^{-1}) estuvo asociada a valores bajos de incorporación de fósforo (menos de 1 fm P cell^{-1} h^{-1}) y máximo contenido en fósforo (9.6 pg P cell^{-1}).

Palabras clave: Actividad fosfatasa alcalina, incorporación de fósforo, *Phaeodactylum tricornutum.*

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INTRODUCTION

Alkaline phosphatase is an enzyme that hydrolyses phosphomonoesters (PME) to be utilised as a potential phosphorus sources for growth. Many microalgae are known to be important producers of alkaline phosphatase (e.g. Berman *et al.*, 1991), especially when they become phosphorus deficient, or conversely, the production is repressed in phosphorus-sufficient algae (Cembella *et al.*, 1984). Some studies suggest that APA can be used as an indicator for phosphorus deficiency (Fitzgerald and Nelson 1966; Berman 1970), although others have questioned this practical approach in natural plankton assemblages (Cembella *et al.*, 1984; Jansson *et al.*, 1988).

The marine diatom *Phaeodactylum tricornutum* is known to exhibit alkaline phosphatase activity (Kuenzler and Perras 1965; Wynne and Rhee 1988). Kuenzler *et al.*, (1963) reported that P-deficient cells could rapidly obtain more phosphorus from natural seawater than was present as phosphate, and concluded that the difference came from dissolved organic phosphorus (DOP) compounds. The enzyme is associated with the cell wall (Flynn *et al.*, 1986). These previous studies have focused in general aspects of short-term increase in phosphorus content when the algae were exposed to different phosphate esters (Kuenzler and Perras 1965), effect of light conditions on enzymatic activity (Wynne and Rhee 1988) or location of the enzyme (Flynn *et al.*, 1986). The present investigation was undertaken to asses the significance of alkaline phosphatase in *Phaeodactylum tricornutum*. The study focuses in the kinetic behaviour of the enzyme, the relative importance of various phosphomonoester as a P source for growth and the relationship between the enzyme activity and total cellular phosphorus.

MATERIAL AND METHODS

Algae and culture conditions

Axenic clonal cultures of *Phaeodactylum tricornutum* Bohlin were obtained from the Instituto de Ciencias Marinas de Andalucía (CSIC) Culture Collection. Stock cultures were grown in f/2 and silicate enriched seawater. Seawater was collected offshore Málaga Bay, prefiltered through 0.22 μm (Nucleopore filters) and stored in a cold room (4 ºC) prior to use. The final medium was filtered again through 0.22 μm size pore filters before the algae were inoculated. Growth was carried out in 2 l flask with a working culture volume of 1 l. The flask was capped and maintained under constant light (130 μmol photon m^{-2} s^{-1}) and temperature (25 ºC) on a rotary shaker. The pH of the cultures was approximately 9. All flasks were autoclaved before use.

Alkaline phosphatase assays

Alkaline phosphatase activity (APA) was routinely assayed in duplicate using both p-nitrophenyl phosphate (*p*NPP) and 4-methylumbelliferyl phosphate (MUP) as substrates. When using the colorimetric method of *p*NPP, phosphatase activity was assayed based on Reichardt *et al.*, (1967). 0.3 ml of 1 mM *p*NPP was mixed with 2.7 ml. Cells were counted in a haemacytometer (type Neubauer). The final substrate concentration was high enough to allow the reaction to proceed at the maximum velocity (see Results). *p*NPP was dissolved in filtered (0.22 μm) natural seawater of phosphate concentration, 0.3 μM collected offshore at Málaga Bay. The seawater was assumed to contain negligible PME concentration as suggested the low total soluble phosphorus (< 0.5 μM). Assays were performed at pH 9. The addition of buffer was not necessary, as pH showed no variations during the assays. After 30-45 min at 25 ºC, continuous light (as above) and gentle shaking, the sample was filtered (0.22 μm) and the absorbance was immediately read at 410 nm against a blank (filtered seawater at pH 9 with substrate solution). Absorbance was converted to concentration using a p-nitrophenol (*p*NP) extinction coefficient of 16800 M^{-1} cm^{-1}, correspondent to pH 9 in seawater (Hernández *et al.*, 1996). This method avoids the addition of NaOH to stop the reaction, which cause precipitation of $Mg(OH)_2$ at high pH (Yelloly and Whitton 1996). The filtering process took less than 3 min and showed insignificant increase in absorbance. APA was expressed as fmol *p*NP $cell^{-1}$ h^{-1}.

When using the fluorimetric method of MUP, phosphatase was measured based on Healey and Hendzel (1979). A fluorescence spectrophotometer (Turner F-2000) were used with filters selected for excitation and emission of 390 and 447 nm respectively. Samples (2.7 ml) were mixed with 0.3 ml of 0.5 mM MUP dissolved in filtered seawater. Again, no buffer addition was required. Samples were incubated as for *p*NPP but with shorter times of incubation (20-30 min), then filtered onto 0.22 μm pore

size filter and fluorescence units read. Fluorescence units were converted to phosphatase units (fmol or fg MU cell^{-1} h^{-1}) from a linear relationship between different methylumbelliferone (MU) concentrations at pH 9 and fluorescence units.

Kinetics parameters

Cells were taken directly from the culture to give 3 10^6 cell ml^{-1} and alkaline phosphatase activity was performed using both *p*NPP and MUP at different concentrations (ranged from 3 µM to 1 mM) to study the kinetics behaviour of the enzyme. Apparent kinetics parameters of the enzyme were computed from two transformations of the Michaelis-Menten equation. The Lineweaver-Burk transformation (1/V vs 1/S) provided maximum velocity by extrapolation of the high-substrate portion of the plot. The apparent half-saturation constants (Ks) were obtained by linear regression from the Eadie-Hofstee transformation (V vs V/S), as in this plot the distribution of errors is more uniform (Price and Stevens 1982). Apparent cooperativity was tested from the slope of a Hill plot ([log V/(Vmax-V)/log S].

Inhibition of phosphatase activity

APA can be either repressed or inhibited by phosphate; the end product of the enzymatic reaction (Cembella *et al.*, 1984). Alternatively, different substrates show different affinities for the enzyme (Pettersson and Jansson 1978). These two effects on phosphatase activity were studied in 75 ml subcultures of *P. tricornutum* of 1.6 10^6 cell ml^{-1}. The cultures were supplied with different concentrations (from 0 to 700 µM) of phosphate or β-glycerophosphate (used as a competitive substrate for MUP) as a source of phosphate. Samples from the different subcultures were collected daily, for four days, to determine APA, using MUP as substrate.

Phosphatase activity and P uptake

The phosphate cleaved from the substrate during the enzymatic reaction is assumed to be taken up by the alga. To test this assumption, the two end-products of the enzymatic reaction were measured essentially as by Hernández *et al.*, (1995). *p*NP (or MU) and P were determined after phosphatase assays performed at substrate concentrations ranging from 0 to 90 µM. Phosphate was determined as soluble reactive phosphorus (SRP) by the sensitive malachite green method (Fernández *et al.*, 1985).

To study the phosphate uptake and phosphatase activity at different phosphorus content in cells of *P. tricornutum,* 1 l of phosphorus depleted subcultures (1.6 10^6 cell ml^{-1}) was supplied with 300 µM phosphate. After four days of incubation, the subcultures was centrifuged (3,500 x g for 5 min), the pellet was washed twice with P-free medium and after a new centrifugation, cells were transferred to a new medium without phosphate. Samples were collected daily to estimate phosphatase activity (using MUP as substrate), total cellular phosphorus, and SRP. Total cellular phosphorus was quantified as soluble reactive phosphorus after an acid digestion (Mackereth *et al.*, 1978) of cells retained on GF/C filters.

Phosphate uptake kinetics were determined at the beginning and end of each experiment based on Healey (1979). 5 ml of sample was centrifuged (3,500 x g for 5 min). Supernatant was drained out

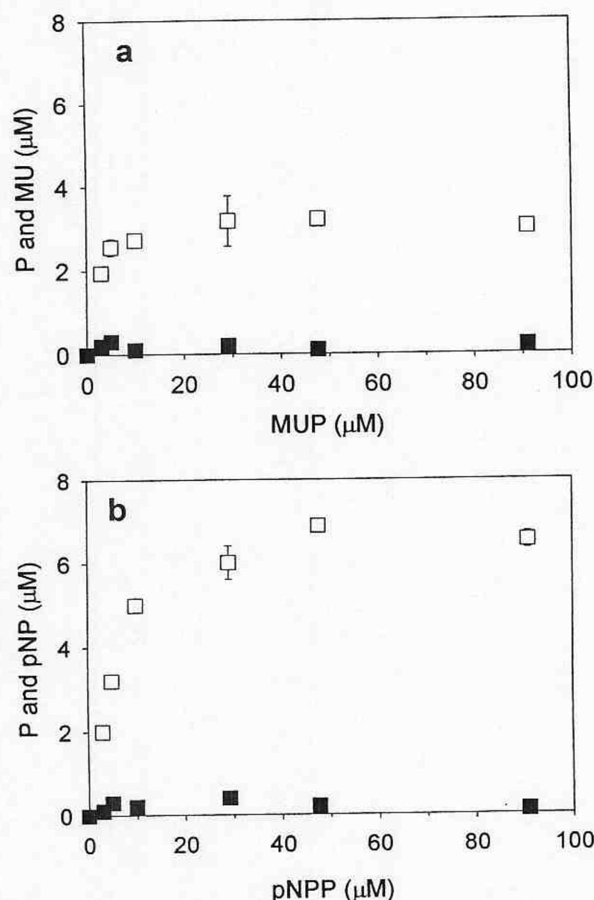


FIG. 1. – Concentrations of the two products of the substrate hydrolysis measured during the assay of alkaline phosphatase activity of *Phaeodactylum tricornutum.* a) Filled squares: phosphate. Open squares: methylumbelliferone. Time of assay: 20 min. b) Filled squares: phosphate. Open squares: p-nitrophenol. Time of assay 60 min. Bars denote SD.

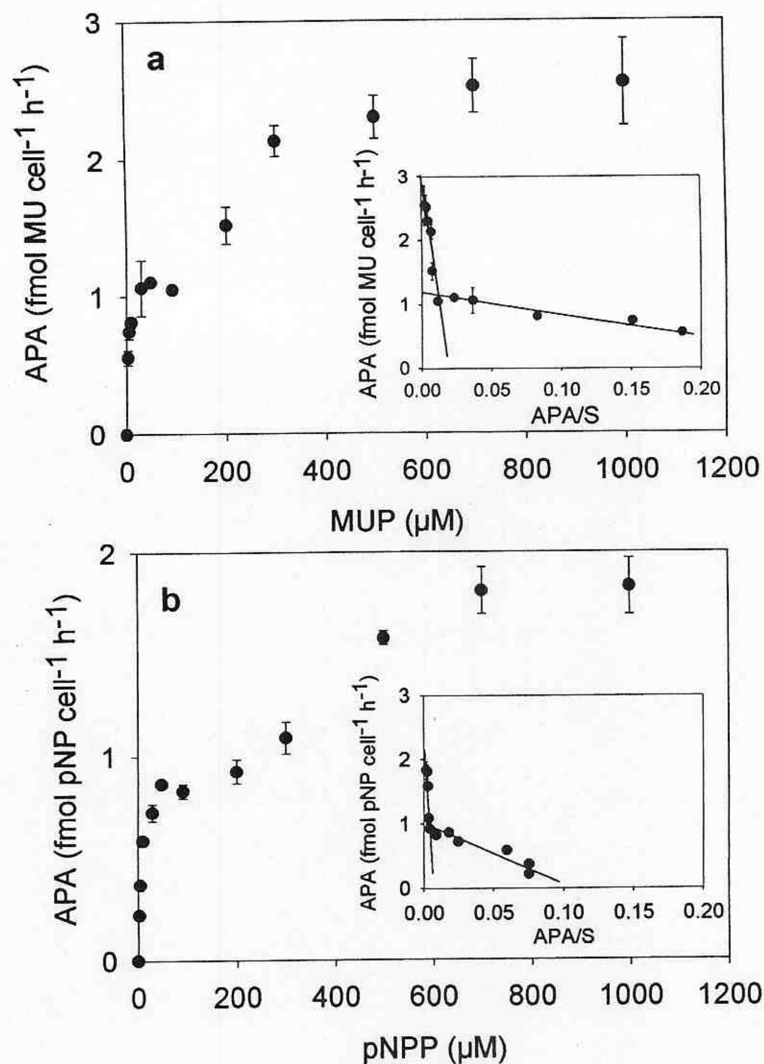


FIG. 2. – *Phaeodactylum tricornutum.* Dependence of velocity of hydrolysis of MUP (a) or *p*NPP (b) on substrate concentration. Inset, repl of the data according to the Eadie-Hofstee plot. An apparent negative cooperativity is suggested by the two straight lines drawn in the figur Bars denote SD.

and cells were incubated in 5 ml of filtered phosphate solution ranging from 2.5 to 50 µM. Then, phosphate concentration was followed during 20 min. Phosphate consumption was linear with time. Therefore, phosphate uptake velocities were estimated from the slope of the fitted straight line of SRP versus time and was expressed as fmol P cell^{-1} h^{-1}.

RESULTS

Phaeodactylum tricornutum phosphatase cleaved efficiently the two model substrates (*p*NPP and MUP) used in the present study (Fig. 1). After incubation time of enzymatic assay, the phosphate concentration in the medium was negligible, suggesting that the phosphoryl moiety was immediately take up by the algae. In contrast, the leaving group (th non-phosphate moiety) remained in solution, show ing the expected saturation kinetic.

Figure 2 shows the dependence of alkaline phos phatase on substrate concentration. A non linear rec iprocal plot was evident either with *p*NPP or MU suggesting the existence of a negative cooperativit for the hydrolysis of the two substrates. Two appar ent Ks values can be deduced from the Eadie-Hofs tee plot (table 1), and can be interpreted in term c apparent negative cooperativity as derived from th slope of the Hill plot, the Hill coefficient nH (tabl 1). This coefficient was nH= 0.48 (r^2= 0.87, p<0.0 for *p*NPP and nH= 0.51 (r^2= 0.85, p<0.05) for MU

The two apparent Ks were lower for MUP tha for *p*NPP. On the other hand, apparent maximu

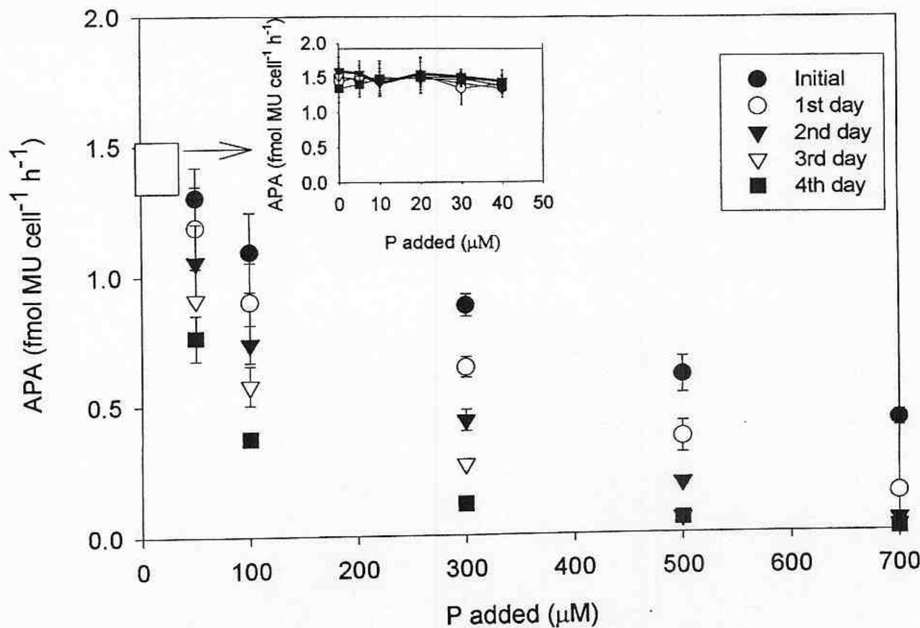


FIG. 3. – *Phaeodactylum tricornutum.* Effect of phosphate addition on alkaline phosphatase activity. The inhibitory effect was tested during four consecutive days of culture under different phosphate concentrations. The independence of the two variables at low phosphate concentration is showed at the inset. Bars denote SD.

velocities were similar, but slightly higher when MUP was used as substrate (Vmax MUP= 3.1 fmol cell^{-1} h^{-1}, Vmax *p*NPP= 2.6 fmol cell^{-1} h^{-1}). Hence, the ratio Vmax/Ks, which may be used as an index of competition in substrate use (Healey 1978) was three times higher with MUP as a substrate (Table 1).

The effect of phosphate addition on phosphate activity is shown in Fig. 3. No significant effect in phosphate activity was observed when cultures were added up to 50 μM phosphate. However, when algae were given higher phosphate concentrations, a gradual decline in activity was observed, being APA virtually suppressed after 4 days of incubation at phosphate concentration > 50 μM. From 100 μM of added phosphate, data were fitted to an exponential function (APA= e^{-bp}, where "b" is the coefficient of decay). At any phosphate concentration, the percentage inhibition depends on the period of incubation, which suggests an inhibition of the enzyme synthesis. This is clearly shown when plotting the coefficient "b" versus the period of incubation (Fig. 4). An inverse linear relationship was evident, suggesting that at phosphate concentrations > 50 μM, the P concentration required for a particular percentage of APA inhibition decreased with time of incubation.

TABLE. 1 – *Phaeodactylum tricornutum.* Apparent semi-saturation constants (ks), maximun velocity (Vmax) and Hill index (nH) exhibited by alkaline phosphatase using MUP and *p*NPP as substrates. Vmax (fm cell-1 h-1) was calculated from the double reciprocal plot (see text). Ks1 and Ks2 (μM) refer to the 'high' and 'low' affinity phases of the enzyme kinetics respectively. The ratio Vmax/Ks was calculated using the apparent high affinity constant (Ks1).

	Ks1	Ks2	Vmax	Vmax/Km	nH
MUP	3.1	176	3.1	0.37	0.51
*p*NPP	8.2	371	2.6	0.11	0.48

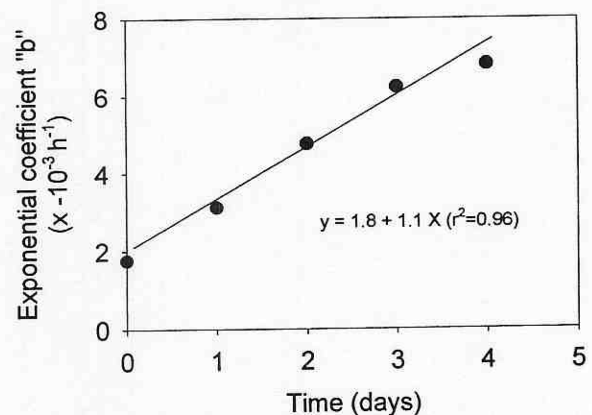
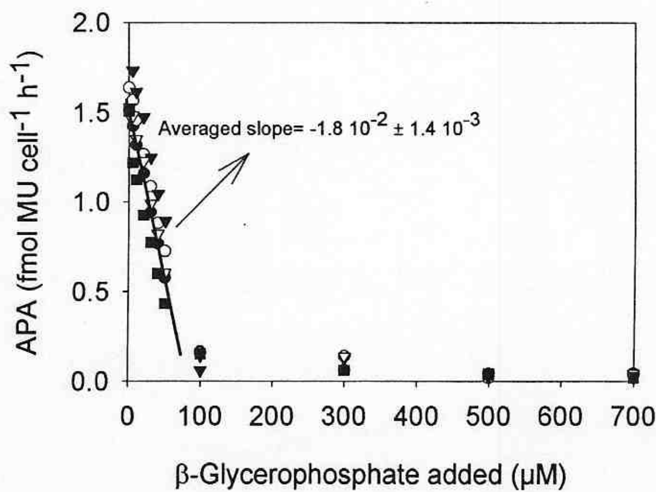


FIG. 4. – *Phaeodactylum tricornutum.* Variation of b, the coefficient of decay in the exponential function fitted in figure 3, during the period of incubation of *Phaeodactylum tricornutum* at phosphate concentration ranging from 50 to 700 μM.



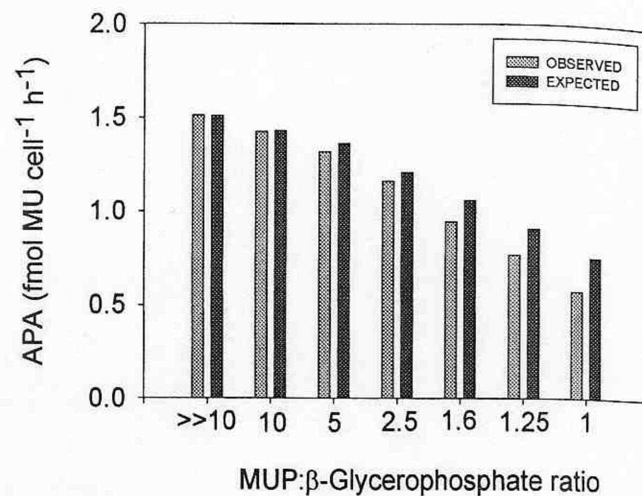


FIG. 5. – *Phaeodactylum tricornutum*. Effect of β-glycerophosphate addition on alkaline phosphatase activity. The decrease in activity (measured with MUP as substrate) was tested during four consecutive days of culture under different β-glycerophosphate concentrations. Symbols as in figure 3.

FIG. 6. – *Phaeodactylum tricornutum*. Effect of β-glycerophosphate addition on alkaline phosphatase. The observed activities are compared with the expected according to the molar MUP/β-glycerophosphate ratios.

In contrast to phosphate addition, β-glycerophosphate suppressed APA (as fmol MU cell^{-1} h^{-1}) at concentration as low as 5 μM (fig. 5). A linear relationship between APA and β-glycerophosphate addition was evident. Besides, 100 μM practically suppressed completely the measured enzymatic activity, and this suppression is similar from the first day of cultivation. We reasoned that if the phosphatase of *P. tricornutum* had the same affinity for MUP and β-glycerophosphate, then the release of phosphate from MUP should decrease by the amount expected for molar dilution. Thus, a molar ratio of 1:1 should decrease the rate of MU production by 50 %. Fig. 6 showed that the decrease in APA using MUP was

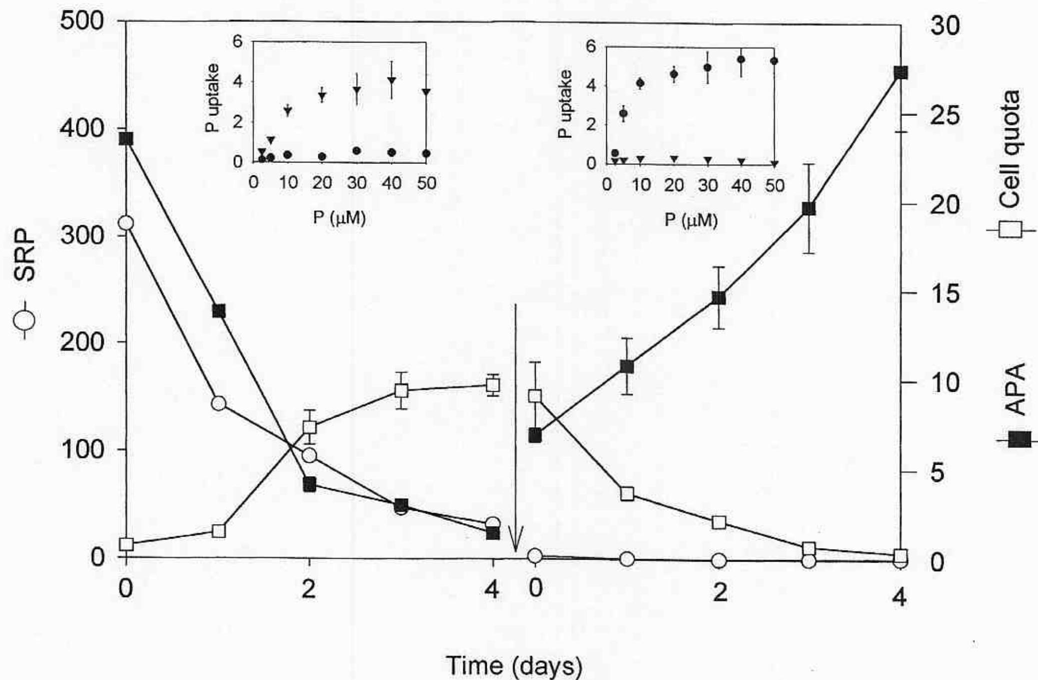


FIG. 7. – *Phaeodactylum tricornutum*. Alkaline phosphatase activity (filled squares), in fg MU cell^{-1} h^{-1}, total cellular phosphorus (open squares), in pg P cell^{-1}, and external phosphate (open circles), in μM, during an experiment started with 300 μM phosphate. After four days (showed by the arrow) of incubations, cells were transferred to a new medium without phosphate. Inset, phosphate uptake, in fmol P cell^{-1} h^{-1}, at the beginning (triangles) and end (filled circles) of the experiment in each culture medium. Bars denote SD.

higher than expected from simple dilution. The difference between the observed and expected phosphatase activity increases with the β-glycerophosphate concentration, suggesting that *P. tricornutum* alkaline phosphatase hydrolysed phosphate preferentially from β-glycerophosphate.

Figure 7 shows the increase in cellular P and the decrease in phosphatase activity (as fg MU $cell^{-1}$ h^{-1}) when P limited cells were cultured in a medium with 300 μM phosphate. At the beginning of the incubation period, cells exhibited high phosphate uptake, showing a Michaelis-Menten kinetics (Vmax=6.5 fm P $cell^{-1}$ h^{-1}, S0.5=6.4 μM, Fig. 7, inset). Then, a marked increase in cellular P content was observed (Fig. 7, left). This increase in P content is parallel to a rapid decrease in cellular APA and phosphate in the culture medium. Maximum internal phosphorus content (9.6 pg P $cell^{-1}$) was reached after 4 days of incubation, when cells did not shown further P uptake (Fig. 7, inlet). Once the cells were transferred to a new P-free medium, the inverse pattern was observed (Fig. 7, right): a constant increase in phosphatase activity and a notable decrease in total cellular phosphorus. After 4 days of incubation in this new conditions, APA was 27 fg MU $cell^{-1}$ h^{-1} and phosphate uptake showed a maximum value of 5 fm P $cell^{-1}$ h^{-1}, slightly lower than the uptake showed at the beginning of the experiment. Cellular P decreased to 0.33 pg P $cell^{-1}$.

APA was inversely related to total cellular phosphorus (Fig. 8). Clearly, the inverse relationship is not linear and although the inverse exponential equation is not fully satisfactory, according to previous investigation (Pettersson, 1980) this is probably the best model to explain the data. The phosphorus cell content showed a threshold value of 9.6 pg P $cell^{-1}$, below which the enzyme activity was triggered. Above this value, APA was low. The maximum APA in *P. tricornutum* (27.3 fg MU $cell^{-1}$ h^{-1}) is reached when the phosphorus content was minimum, and enzyme synthesis was virtually suppressed when internal phosphorus was higher than 10 pg P $cell^{-1}$.

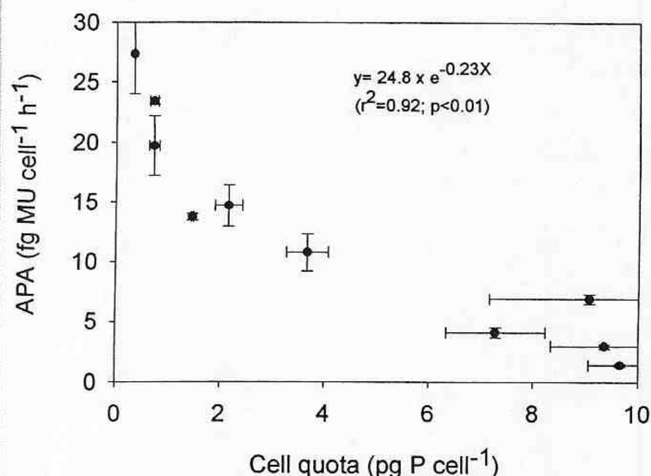


FIG. 8. – *Phaeodactylum tricornutum*. Relationship between alkaline phosphatase activity and total cellular phosphorus.

DISCUSSION

Phaeodactylum tricornutum is capable of using several sources of organic P for growth. The two model substrate used here (*p*NPP and MUP) showed that phosphate was taken up immediately after substrate cleavage. The uptake occurs even at low (< 5 μM) PME concentration suggesting that the algae may utilise DOP at environmental concentration likely to occur under natural conditions. The use of β-glycerophosphate gave a quite similar growth rate to that obtained with inorganic P. Other microalgae have shown the same phenomenon (Berman *et al.*, 1991; Van Boekel 1991), although the growth rates depended on the DOP source. DOP compounds may constitute a significant proportion of the total dissolved phosphorus in marine waters (Banoub and Williams 1972, Thingstad and Rassoulzadegan 1995), and some appear to cycle rapidly (Azam *et al.*, 1990), which underlines their potential importance for microalgal growth. In contrast to the present study, Rivkin and Swift (1980) in the dinoflagellate *Pyrocystis noctiluca* and Hernández *et al.*, (1995) in red algae suggested that some of the phosphate cleaved from PME remains in solution and might be available to other microorganism.

Most of the activity observed in the present study was associated with cells, as dissolved phosphatase activity was always lower than 7 % of the total. Flynn *et al.*, (1986) showed that *P. tricornutum* has a cell-bound alkaline phosphatase and a free dissolved or extracellular alkaline enzymes (Price and Morel 1990), but the term extracellular used here refers to the dissolved activity. The dissolved activity found in that study may account for 30 % of the total APA at pH 10. According to Flynn *et al.*, (1986), the activity found extracellularly may come from the same enzyme that was not firmly bound to the cells. Electrophoretic studies are needed to examine this problem.

APA showed an apparent negative cooperativity for the two model substrates assayed here. This negative cooperativity is scarcely reported in previous works, as usually alkaline phosphatase of phytoplankton exhibits a typical Michaelis-Menten kinetics (Wynne and Rhee 1988; Lubian *et al.*, 1992). Rivkin and Swift (1980) found an apparent negative cooperativity (they named it multiphasic substrate dependent APA) in the dinoflagellate *Pyrocystis noctiluca* and this cooperativity has also been found in APA of bacteria (Lazdunski 1972) or macroalgae (Hernández *et al.*, 1995). Ks values are affected by the range of substrate concentrations over which measurements are made (McComb *et al.*, 1979), and this might be an explanation for this seeming disparity of kinetics. The ecological significance of the apparent negative cooperativity found in *P. tricornutum* lies in the linkage of enzymatic activity to external substrate concentration (Cembella *et al.*, 1984), so that the algae would assure a regular P supply form PME regardless of the external substrate concentration. The high Vmax may allow rapid uptake in the presence of transient DOP pulses, which are probably common in many natural environments (Islam and Whitton 1992). On the other hand, the lower Ks might assure a high affinity for substrates in case of low PME concentrations.

The kinetic parameters of alkaline phosphatase varied with the substrate structure, as the enzyme is not substrate specific. Usually, alkaline phosphatase shows higher affinity for MUP than for *p*NPP (Whitton *et al.*, in press), suggesting that MUP is a better substrate to study the significance of APA. The lower Ks value of *P. tricornutum* phosphatase was similar to the lower Ks given by Rivkin and Swift (1980). Previous Ks values estimated in *P. tricornutum* (using *p*NPP) were similar to the lower Ks value reported here (Wynne and Rhee 1988) or between the two Ks values (Flynn *et al.*, 1986). Also Vmax was similar for the values reported by Wynne and Rhee (1988) also in *P. tricornutum* or by Lubian *et al.*, (1992) in the phytoplankters Nannochloris and Nannochloropsis.

The high external phosphate concentrations necessary to decrease APA suggests that under environmental conditions, APA of *P. tricornutum* may vary in response to internal phosphatase rather than external phosphate. The decrease in activity was observed under high external phosphate levels, rarely found in nature. An inverse relationship between APA and total cellular phosphorus has been reported previously (Wynne 1977 Pettersson 1985; Hernández *et al.*, 1993). This relationship strongly suggests that in *P. tricornutum* APA may be used as an indicator of phosphorus limitation.

The maximum values of cellular phosphorus in *P. tricornutum* cells (9.6 pg P cell^{-1}) were highe than previous values reported in the literature Kuenzler and Perras (1965) found a quantity o phosphorus per *Phaeodactylum* cell of 0.6 pg P bu still in cells not saturated whereas Kuenzler an Ketchum (1962) reported a maximum concentratio of 2 pg cell^{-1}. However, the concentrations were similar to those found in the cyanobacterium *Anabaena flos-aquae* (Thompson *et al.*, 1994). These discrepancy may be due to a large storage capacity of th clone used or the different physiological status o the cells.

In conclusion, the results suggest that *P. tricornutum* can use different PMEs as a P source fo growth. However, some difficulties exist in transforming the results obtained with cultured algae t the marine ecosystems. The extent to which *P. tricornutum* may use PME will ultimately depend o its concentration and its composition in relation t the substrate spectrum of the algae phosphatase, a neither MUP nor *p*NPP are natural components o DOP. Future studies are also needed to estimate th rate at which PME is cleaved in relation of the tot P demand of the algae.

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