Journal of Phytology 2009, 1(2): 75–85
© *Journal of Phytology, 2009*

ISSN: 2075-6240
Available Online: www.journal-phytology.com

REGULAR ARTICLE

SIZE DIFFERENTIAL GROWTH AND UPTAKE KINETICS OF INORGANIC PHOSPHATE IN SOME MARINE DIATOMS

N. Ashok Kumar[1,2], V.N. Raja Rao[1], R. Rengasamy[1]

[1]*Centre for Advanced Studies in Botany, University of Madras, Guindy campus, Chennai - 600 025, Tamil Nadu, India*

[2]*Department of Botany, Arignar Anna Government Arts College, Villupuram – 605 602, Tamil Nadu, India*

SUMMARY

The marine diatoms such as *Amphiprora gigantea* O'Meara, *Amphora coffeaeformis* (Agardh) Kütz., *Cocconeis heteroidea* Hantz and *Cyclotella meneghiniana* Kütz. isolated from the coastal waters were made axenic and investigated for their growth, kinetics of phosphate uptake and assimilation. Phosphate-phosphorus at higher concentration depressed growth and division rates of all the diatoms. The uptake and assimilation of phosphate-phosphorus followed the classic Michaelis-Menten kinetics. Dark uptake was 37-71% when compared to light saturated uptake. *Amphiprora gigantean*, the largest diatom showed the low K_s and K_m values whereas the smallest diatom *Cyclotella meneghiniana* exhibited high K_s and K_m values for phosphate uptake and assimilation. DCMU inhibited phosphate uptake even at 2.5µM concentration indicated that the phosphate uptake is mediated mainly by the energy derived from photosynthesis.

Keywords: Phosphate uptake; marine diatoms; assimilation; inhibition.

N. Ashok Kumar. et al. Size differential growth and uptake kinetics of inorganic phosphate in some marine diatoms.J Phytol 1 (2009) 75-85

***Corresponding Author**, *Email*: thamiz@gmail.com

1. Introduction

Apart from nitrogen, phosphorus is one of the major macronutrients which plays a vital role in the growth and reproduction of algae. It also limits primary production in the aquatic habitats. In nature, inorganic phosphorus is present mainly as orthophosphate and is taken up in the same form by phytoplankton. The important phenomenon for many algae is that they are known to accumulate and store large quantities of phosphate as polyphosphate when grown under phosphate enrichment [1,2,3,4]. Polyphosphate serves as the immediate phosphorus source for the growing algae [5] and also serves as a main source during phosphate starvation [6].

Generally, phosphorus uptake is more in P-deficient cells than in P-replete cells [7]. Rosenberg et al. [8] have found that in P-starved cells, the P-uptake rate is much faster than in non-starved cells until a primary pool has been filled. Even after that, phosphate continues to pass through the pool for cellular utilization till the pool reaches the level close to external phosphate concentration. Because

the specific growth rate of planktonic algae depends on internal rather than external nutrient levels [9], the stored internal phosphate supports growth even after phosphate is exhausted in the medium.

Phosphate incorporation occurs in three steps, first phosphate is transported into cell by electroneutral protein phosphate transporter, then this transported phosphate is converted to ATP and finally polyphosphates are formed from ATP [10]. The number of cells per unit area also could influence the phosphate uptake in a cyanobacterium *Anacystis nidulans*. When more cells were exposed to phosphate, the uptake was found faster [6]. Further, each cell could adapt itself to the concentration changes caused by the whole population. When two different dilutions of *Anacystis nidulans* were given a high phosphate pulse, the decrease in the external phosphate was faster in the high density suspension [11].

Nitrogen or phosphorus uptake by phytoplankton is influenced by a number of factors including their size, shape, mobility and intracellular processes in addition to the physico-chemical characteristics of the external environment [12]. The surface area/volume characteristic of phytoplankton cells has been considered to determine nutrient assimilation properties and hence has a direct bearing on their growth and metabolism. Such an influence is not surprising since the cell surface defines the maximum area across which nutrients can pass through and light energy can be absorbed. This dependence of growth and subsistence quota on cell size implies that cell surface area influences a variety of underlying metabolic processes, the most notable of these being nutrient uptake. This present study deals with the influence of cell surface area and cell volume on the uptake and assimilation of phosphate in different sized phytoplanktons.

2. Materials and methods

The diatoms *Amphiprora gigantea* O'Meara, *Amphora coffeaeformis* (Agardh) Kütz., *Cocconeis heteroidea* Hantz and *Cyclotella meneghiniana* Kütz. were isolated from the different habitats (Table 1). They were made axenic by antibiotic treatment following the method described by Droop [13] and maintained in Guillard's F/2 medium [14] at 24±1°C in a thermostatically controlled room and illuminated with cool white fluorescent lamps providing an irradiance of 40 µE/m2/s in a 12:12 light dark regime. The diatoms were identified with the help of a standard manual and a local flora [15,16]. The mean cell surface and volume of these diatoms were calculated using the formula given by Hillebrand et al. [17] and are represented in Table 2.

Table 1. Diatom cultures used in the present study

Diatoms	Collection site	Month and year of isolation
Amphiprora gigantea	Foreshore Estate, Chennai	October 1999
Amphora coffeaeformis	Near shore temple, Mahabalipuram	September 1999
Cocconeis heteroidea	Foreshore Estate, Chennai	September 1999
Cyclotella meneghiniana	Coastalarea, Kovelong	October 1999

Growth measurement

Growth of the diatoms was recorded by counting the cells using a `Neubauer' haemocytometer. Growth curves were plotted against time from log10 of cell number taken on every alternate days till 12th day. A straight line fitted through points corresponding to exponential phase, division rates were calculated using the formula:

$$\text{Division rate} = \frac{\text{Log}_{10}\text{ final} - \text{Log}_{10}\text{ initial}}{\text{`t' (days)} \times 0.301}$$

The amount of phosphate phosphorus was estimated by following the method of Murphy and Riley [18]. The acid phosphatase activity and alkaline phosphatase activity were assayed by the method described by Baker and Takeo [19] and Malamy and Horecker [20], respectively.

A linear transformation of the michaelis-menten equation was used to determine the values of μ_{max}, V_{max}, Kgs, K_s and K_m. They were calculated by linear regression analysis using a statistical programme "Hyperbolic regression analysis" based on the equation $S/v = (1/V_{max}) S + (K_s/V_{max})$ with S/v as the ordinate and S as the abscissa [21]. In this transform, Kgs, Ks and Km are given as the negative x-intercept and $1/V_{max}$ as the slope of the regression equation of S on (S/v).

3. Results

Among the four diatoms, *Cyclotella meneghiniana* showed the lowest surface area and cell volume, whereas *Amphiprora gigantea* showed the highest. *Amphora coffeaeformis* showed a lower cell surface area but a higher cell volume, whereas *Cocconeis heteroidea* showed moderate cell surface area and cell volume (Table 2).

Table 2. Mean cell surface area and volume of the diatoms

Diatoms	Cell surface area μm^2	Volume μm^3
Amphiprora gigantea	2921	10,357
Amphora coffeaeformis	795	3090
Cocconeis heteroidea	1233	2735
Cyclotella meneghiniana	650	1294

Growth of diatoms in different concentrations of Phosphate

The phosphate in the diatoms were first depleted by growing them for 4 days in P-free F/2 medium and then inoculated into F/2 medium amended with different concentrations of NaH_2PO_4 ranging from 0.006 mM to 0.48 mM. Cell counts were taken on every alternate days up to 12th day. Data are presented as division rates in table 3. Substrate concentrations v_s growth rates (S/μ) plotted against nutrient concentrations (S) resulted a straight line and the point of intersection of this straight line with the axis indicated the half-saturation constant for growth. μ_{max} and half saturation constants for growth (K_{sg}) for NaH_2PO_4 were also obtained (Fig. 1 and Table 4).

Table 3. Division rates (divisions/day) of diatoms grown in different concentrations of NaH_2PO_4

Diatoms	Concentration of NaH_2PO_4 in mM				
	0.006	0.03	0.09	0.19	0.48
Amphiprora gigantea	0.67	0.91	0.97	0.92	0.61
Amphora coffeaeformis	0.64	0.98	1.01	0.95	0.75
Cocconeis heteroidea	0.58	1.05	1.10	1.08	0.76
Cyclotella meneghiniana	0.78	1.16	1.20	1.02	0.72

Table 4. μ_{max} and K_{sg} for diatoms grown in different concentrations of NaH_2PO_4

Diatoms	μ_{max}	K_{sg} (mM)
Amphiprora gigantea	1.00	0.0030
Amphora coffeaeformis	1.05	0.0031
Cocconeis heteroidea	1.17	0.0045
Cyclotella meneghiniana	1.24	0.0030

All the diatoms showed high division rates up to 0.19mM of phosphate, above which division rates decreased.

Among the four diatoms, *Amphiprora gigantea* showed the lowest μ_{max} and *Cyclotella meneghiniana* the highest. All of them showed similar K_{sg} values except *Cocconeis heteroidea* whose K_{sg} was slightly higher.

KINETICS OF PO4- UPTAKE

Determination of V_{max} and K_s

Cultures raised in F/2 medium were inoculated into P-free F/2 medium amended with 5 µM of NaH_2PO_4 and incubated for 3 days. At the end of third day, the medium did not know to contain any phosphate-phosphorus these cultures were employed in short term and long term uptake studies. Experiments were carried out with different concentrations of PO_4- in both light and dark conditions. Velocity of PO_4- uptake (v) was plotted against substrate concentration S and from the hyperbolas V_{max} values were obtained. S/v Vs S plots were also obtained to find out the Ks values (Fig. 2). Among the diatoms, *Amphiprora gigantea* showed a low Ks value, whereas *Amphora coffeaeformis* and *Cyclotella meneghiniana* showed high Ks values (Table 5). Both *Amphiprora gigantea* and *Cyclotella meneghiniana* showed a higher capacity for dark uptake compared to the other two diatoms.

Table 5. Vmax and Ks for PO4- uptake by diatoms in Light and Dark

Diatoms	Mean cell surface area (μm^2)	Mean cell volume (μm^3)	Ks µM (Light)	Vmax (n moles/106 cells/h)		
				Light	Dark	% of Inhibition
Amphiprora gigantea	2921	10,357	6.83	215.10	153.10	28.82
Amphora coffeaeformis	795	3090	16.57	150.8	51.77	65.67
Cocconeis heteroidea	1233	2735	14.04	148.9	55.84	62.50
Cyclotella meneghiniana	650	1294	17.47	144.2	97.15	32.63

Effect of metabolic inhibitors on PO_4- uptake by diatoms

Short term uptake experiments were carried out at 20 µM PO4- concentration in the presence of KCN and DCMU (Table 6). Even at very low concentration (2.5 µM) of DCMU inhibited PO_4- uptake by 40-50%, while KCN inhibition of PO_4- uptake was low even at 1.0 µM concentration.

Table 6. Effect of metabolic inhibitors on PO_4- uptake by different diatoms

Diatoms	Phosphate uptake (v) rates (n moles/106 cells/h)				
	Control (Velocity)	Inhibitors			
		KCN (1.0 mM)		DCMU (2.5 µM)	
		Velocity	% of inhibition	Velocity	% of inhibition
Amphiprora gigantea	159.2	130.8	17.8	82.3	48.3
Amphora coffeaeformis	87.5	82.6	5.6	58.5	33.1
Cocconeis heteroidea	85.3	71.4	16.3	48.5	43.1
Cyclotella meneghiniana	80.2	62.4	22.2	45.8	49.4

Long term PO_4- uptake

Long term PO_4- uptake (hours)

The diatoms were initially grown in the P-free basal medium amended with only 5µM of PO_4- for a period of three days in order to achieve P-depleted cells. Then the medium was enriched with 50µM of PO_4-P. At an every interval of 30 minutes, 10 mL of culture was taken and centrifuged. The cell free supernatant was analyzed for PO_4- remaining in the medium. Among the four diatoms, *Cyclotella meneghiniana* and *Amphora coffeaeformis* showed a faster PO_4- uptake rate than the other two (Fig. 3).

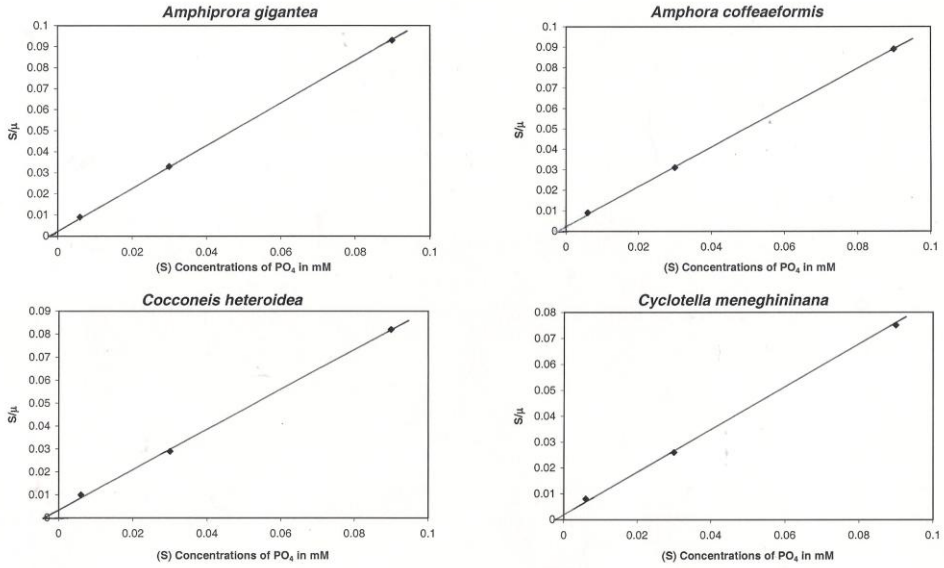

Fig.1 Half-saturation constants for growth (K_s^g) of diatoms grown in NaH_2PO_4

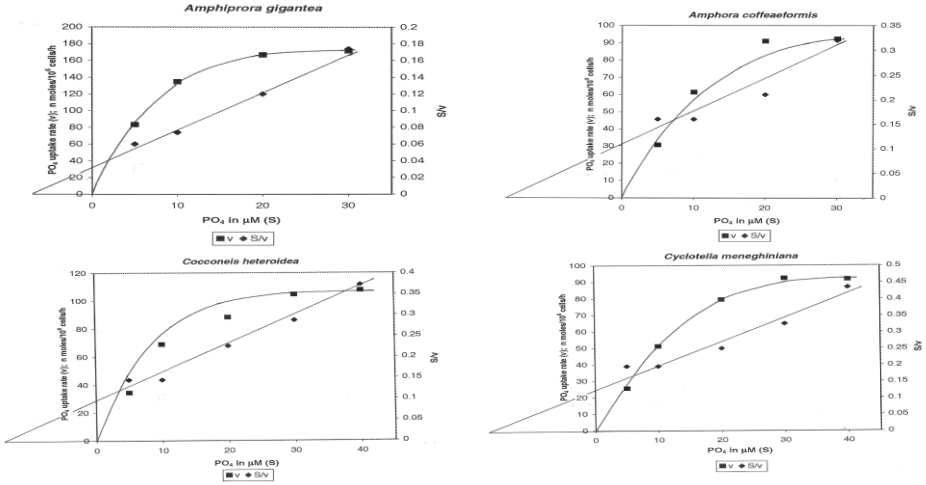

Fig. 2 Phosphate uptake rates (v) of different diatoms. Half-saturation constant (Ks) is given as the negative S-intercept of the linear regression of (S/v) Vs S

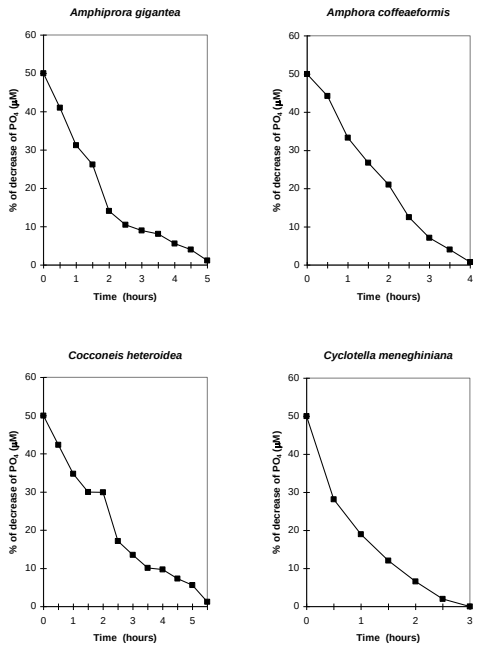

Fig. 3 Long term uptake of phosphate in diatoms (hours)

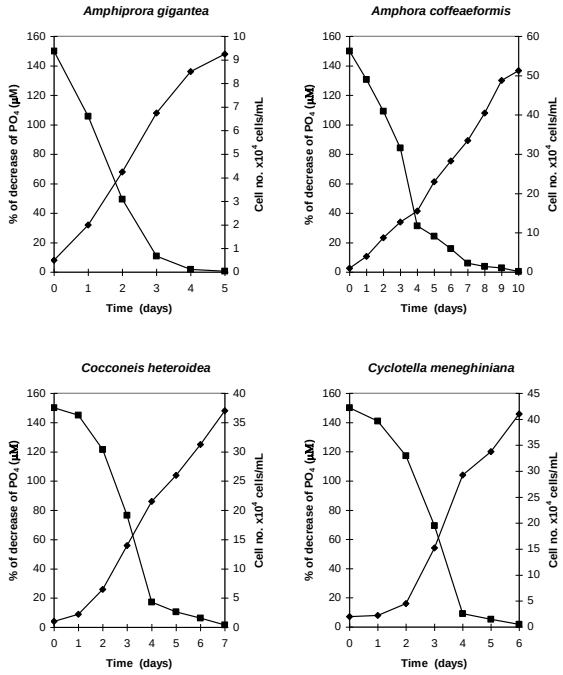

Fig. 4 Long term uptake of phosphate by different diatoms (days)

Long term PO4- uptake (days)

The P-depleted cells obtained in the previous experiment were also taken for this study. The medium was enriched with 150µM of PO_4-. Every 24 hours 10 mL of culture was taken and centrifuged, and the supernatant was analyzed for PO_4- remaining in the medium. The increase in cell number was found coinciding to the decrease of PO_4- in the medium. The rate of PO_4- uptake was greater in *Amphiprora gigantea* than the other diatoms, *Amphora coffeaeformis* showed the least uptake (Fig. 4).

Enzyme kinetics

Acid and alkaline phosphatases

Determination of V_{max} and K_m

Enzymes involved in the assimilation of phosphate namely acid phosphatase and alkaline phosphatase were assayed in diatom cultures grown for four days in 1 mg/L NaH_2PO_4 amended F/2 medium. Both acid and alkaline phsophtase activities were estimated at different substrate concentrations (P - NPP). The assay mixture was incubated at 24 ± 1°C for 10 - 15 minutes. Activity was calculated as n moles of P-NPP hydrolyzed per 106 cells per hour. Lineweaver - Burk Plots were used to determine K_m and V_{max} values (Table 7). All the diatoms showed a higher V_{max} and K_m values for acid phosphatase than for alkaline phosphatase. The Km value for acid phosphatase in *Amphiprora gigantea* was lower than for alkaline phosphatase.

Table 7. Comparison of Vmax and Km for acid and alkaline phosphatases

Diatoms	Acid phosphatase		Alkaline phosphatase	
	V_{max} n moles of P-NPP hydrolyzed / 106 cells/h	Km in (mM)	Vmax n moles of P-NPP hydrolyzed / 106 cells/h	Km in (mM)
Amphiprora gigantea	258.1	0.12	216.4	0.15
Amphora coffeaeformis	356.8	0.65	280.9	0.18
Cocconeis heteroidea	362.9	0.37	99.2	0.08
Cyclotella meneghiniana	243.0	0.38	209.5	0.24

Effect of metabolic inhibitors on acid and alkaline phosphatases

The activities of both acid and alkaline phosphatase were assayed in the diatoms grown in 1 mg/L NaH_2PO_4 amended F/2 medium with 100µM of P-NPP in the presence of KCN (1mM) and DCMU (5µM) (Table 8).

Table 8. Effect of inhibitors on phosphatase activity (n moles of P-NPP hydrolyzed/106 cells/h)

Diatoms	Acid phosphatase			Alkaline phosphatase		
	Control	KCN (1mM)	DCMU (5µM)	Control	KCN (1mM)	DCMU (5µM)
Amphiprora gigantea	121.88	0 (100)	15.23 (87.50)	96.00	0 (100)	8.00 (91.67)
Amphora coffeaeformis	40.00	0 (100)	3.33 (91.67)	104.35	0 (100)	20.87 (80.00)
Cocconeis heteroidea	92.63	0 (100)	12.62 (86.38)	54.85	0 (100)	9.14 (83.34)
Cyclotella meneghiniana	48.00	0 (100)	8.00 (83.33)	56.21	0 (100)	8.65 (84.61)

(Values in parentheses denote % reduction over control)

KCN completely inhibited both acid and alkaline phosphatase activities. But DCMU inhibited the activities by 80 - 92%.

4. Discussion

The division rates of the diatoms namely *Amphiprora gigantean, Amphora coffeaeformis, Cocconeis heteroidea* and *Cyclotella meneghiniana* were found low at low phosphate (NaH_2PO_4) concentration, but the rates increased as the phosphate concentrations of the medium increased. However the concentrations above 0.09mM decreased division rates slightly.

Falkner et al. [22] explained that when the available phosphate is low, it will be useful for slow growth of the organisms because they utilize the stored phosphate economically over a prolonged period. However, if the cells are loaded with a high amount of phosphate in large polyphosphate granules, it is advantageous for the organism to proliferate as fast as possible under the prevailing ecological conditions.

All the four diatoms investigated in the present study showed very low K_sg values for phosphate implying that very low phosphate concentration was found sufficient to produce maximum division rates in these diatoms and K_sg of PO_4- was not size dependent. In the case

of uptake, the smallest diatom *Cyclotella meneghiniana* showed a very high Ks value and a low V_{max} value. Whereas the largest diatom Amphiprora gigantea showed a very low Ks and a high V_{max} value for PO_4- uptake. Similarly in the long term uptake *Amphiprora gigantea* showed faster uptake of PO_4- than other diatoms indicated that it was capable of utilizing phosphate even at low concentration. Wagner et al. [23] found that during phosphate limited growth, the synthesis of phosphate binding protein, a constituent of cytoplasmic membrane, was induced. Under low phosphate condition the TcPHO (high affinity phosphate transporter gene) mRNA level increased considerably when compared to P-replete culture. However, the addition of phosphate to low phosphate culture effectively inhibited TcPHO mRNA expression [24]. In the present study the high uptake of PO4- by *Amphiprora gigantea* is due to its larger size and it may produce a large amount of phosphate binding protein. Thus it showed a high PO4- uptake and presumably TcPHO gene may be induced to high level.

In algae, the most important effect on phosphate uptake is the action of light, because phosphorylated compounds are closely involved in metabolic and energy transforming reactions of photosynthesis. Many experiments with different algae have proved that phosphate uptake and its incorporation is greater in light than in dark. But Riegman et al. [25] reported that the affinities for phosphate uptake in the dark and in light are similar. At high growth rate, the affinity of `P' uptake in dark increased by a factor of two or even more. In the present study, all the four diatoms utilized PO_4- in dark but compared to light the uptake in dark was very much reduced (28.82-65.67%) to that of light saturated uptake implying that energy derived from photosynthesis is necessary for the uptake of PO_4-.

Both KCN and DCMU inhibited P-uptake rates of which DCMU inhibited PO_4-uptake even at 2.5µM concentration indicated that energy input from both photosynthesis and respiration seemed necessary for maximum PO_4- uptake. Similar results have been obtained by Rivkin and Swift [7] where addition of DCMU caused a gradual delay in the rate of PO_4- uptake to the extent of 60-70% of the uninhibited control. Graziano et al. [26] pointed out that the addition of DCMU blocked the transfer of electrons causing the effect.

Alkaline phosphatase activity is one of the most commonly used indicators of P-deficiency. Graziano et al. [26] stated that alkaline phosphatase activity is induced only in the starved state. Alkaline phosphatase is synthesized mainly for the hydrolysis of organic P compounds and polyphosphate bodies to maintain an adequate supply of P.

In the present study, Km values for acid phosphatase and alkaline phosphatase were several folds greater than the Ks values for the uptake. V_{max} for acid phosphatase was higher for all the diatoms when compared to Vmax for alkaline phosphatase. In contrast, the Km values for alkaline phosphatase were found very low compared to acid phosphatase. However, *Amphiprora gigantea* showed a similar Km values for both the enzymes. Therefore, *Amphiprora gigantea* had the high capacity for P-uptake and its incorporation between the pH 5.6-8.0. Table 9 shows a comparison of Ks and Km values for uptake and assimilation of phosphate obtained in the present study with those published earlier.

Table 9. Ks and Km values for uptake and assimilation of PO_4- by various algae

Organisms	Ks (μM)	Km (mM)		Reference
		acid phosphatase	alkaline phosphatase	
Thalassiosira fluviatilis	1.72	-	-	[27]
Thalassiosira Pseudonana	0.58	-	-	[27]
Thalassiosira pseudonana	0.67	-	-	[28]
Navicula pelliculosa	5.12-11.75	-	-	[29]
Monochrysis lutheri	0.51	-	-	[30]
Olisthodiscus luteus	1.50	-	-	[31]
Amphora coffeaeformis	54.00	111.10	142.86	[32]
Navicula pelliculosa	37.50	400.00	71.42	[32]
Thalassiosira fluviatilis	30.00 & 204.00 (Biphasic)	500.00	250.00	[32]
Amphiprora gigantea	6.83	0.12	0.15	Present study
Amphora coffeaeformis	16.47	0.65	0.18	Present study
Cocconeis heteroidea	14.04	0.37	0.08	Present study
Cyclotella meneghiniana	17.47	0.38	0.24	Present study

It was hypothesized that the algal P-status is likely to be important in the regulation of enzymatic synthesis [33]. Among the two inhibitors tested, KCN completely inhibited both the acid and alkaline phosphatase activities, whereas DCMU inhibited 80-92% of the enzyme activities.

Acknowledgements

The authors wish to thank the Director, Centre for Advanced Studies in Botany, University of Madras, for his keen interest and for providing the facilities. One of the authors (N. Ashok Kumar) thanks the University Grants Commission, New Delhi, India for the finical assistance.

References

1. Callow, M.E. and Evans, L.V. 1979. Polyphosphate accumulation in sulphur-starved cells of *Rhodella maculata*. Br. J. Phycol., 14: 327-337.

2. Elgavish, A. and Elgavish, G.A. 1980. 31P-NMR differentiation between intracellular phosphate pools in *Cosmarium* (Chlorophyta). J. Phycol., 16: 368-374.

3. Elgavish, A., Elgavish, G.A., Halmann, M. and Berman, T. 1980. Phosphorus utilization and storage in batch cultures of the dinoflagellate *Peridinium cinctum*. F.Westii. J. Phycol., 16: 626-633.

4. Jacobson, L. and Halmann, M. 1982. Polyphosphate Overplus Phenomenon in *Plectonema boryanum*. Can. J. Microbiol., 20: 1235-1239.

5. Bonotto, S., Lurquin, P. and Mazza, A. 1976. Recent Advances in research on the marine alga *Acetabularia*. In Russell, R.S. and Yonge (Eds.) Advances in marine biology. Academic press, London, 14: 124-205.

6. Wagner, F., Sahan, E. and Falkner, G. 2000. The establishment of coherent phosphate uptake behaviour by the cyanobacterium *Anacystis nidulans*. Eur. J. Phycol., 35: 243-253.

7. Rivkin, R.B. and Swift, E. 1982. Phosphate uptake by the oceanic dinoflagellate *Pyrocystis noctiluca*. J. Phycol., 18: 113-120.

8. Rosenberg, H., Medveczky, N. and La Nause, J.M. 1969. Phosphate transport in Bacillus cereus. Biochem. Biophys. Acta, 193: 159-167.

9. Rhee, G.Y. 1973. A continuous culture study of phosphate uptake, growth rate and polyphosphate in *Scenedesmus* sp. J. Phycol., 9: 495-506.

10. Falkner, G., Wagner, F. and Falkner, R. 1994. On the relation between phosphate uptake and growth of the Cyanobacterium *Anacystis nidulans*. Microbiol., 317: 534-541.

11. Falkner, G., Wagner, F., Small, J.V. and Falkner, R. 1995. Influence of fluctuating phosphate supply on the regulation of phosphate uptake by the blue-green alga *Anacystis nidulans*. J. Phycol., 31: 745-753.

12. Cochlan, W.P. and Bronk, D.A. 2001. Nitrogen uptake kinetics in the Ross sea, Antarctica. Deep-Sea Res., II 48: 4127-4153.

13. Droop, M.R. 1967. A procedure for routine purification of algal culture with antibiotics. Br. Phycol. Bull., 3: 295-297.

14. Guillard, R.R.L. and Ryther, J.H. 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve). Gran. Can. J. Microbiol., 8: 229-239.

15. Hustedt, F. 1927-1966. Die Kieselagen Deutschlands, Österreiehs Und der Scheweiz. In Rabenhorst's Kryptogamen- Flora. 7: 1-920 (1927-1930); 7: 1-845 (1931-1959); 7: 1-816 (1961-1966).

16. Subrahmanyan, R. 1946. A systematic account of the marine planktonic diatoms of the madras coast, Proc. Indian Acad. Sci., 24(4): 85-197.

17. Hillebrand, H., Durselen, C.D., Kirschtel, D., Pollingher, U. and Zohary, T. 1999. Biovolume calculation for pelagic and benthic microalgae. J. Phycol., 35: 403-424.

18. Murphy, J. and Riley, J.P. 1962. A modified single solution method for the determination of phosphate in natural waters. Anal. Chem. Acta, 27: 31-36.

19. Baker, J.E. and Takeo, T. 1973. Acid phosphatase in plant tissues. 1. Changes in activity and multiple forms in tea leaves and tomato fruit during maturation and senescence. Plant Cell Physiol., 14: 459-471.

20. Malamy, M.H. and Horecker, B.L. 1964. Methods of enzymatic analysis. Bergmer, H.U. (Ed.) Hans Ulrich Academic Press Inc., 9: 639.

21. Eppley, R.W., Rogers, J.N. and McCarthy, J.J. 1969. Half-saturation constants for uptake of nitrate and ammonium by marine phytoplankton. Limnol. Oceanogr., 14: 912-920.

22. Falkner, G., Wagner, F. and Falkner, R. 1996. The Bioenergetic coordination of a complex biological system is revealed by its adaptation to changing environmental conditions. Acta. Biotheoretica, 44: 283-299.

23.Wagner, F., Gimona, M., Ahorn, H., Peschek, G.A. and Falkner, G. 1994. Isolation and functional reconstitution of a phosphate binding protein of the cyanobacterium *Anacystis nidulans* induced during phosphate-limited growth. J. Biol. Chem., 269(8): 5509-5511.

24.Chung, C.C., Hwang, S.P.L. and Chang, J. 2003. Identification of a high affinity phosphate transporter gene in a Prasinophyte alga, *Tetraselmis chui*, and its expression under nutrient limitation. Appl. Envir. Microbiol., 69(20): 754-759.

25.Riegman, R., Stolte, W., Noordeloos, A.A.M. and Slezak, D. 2000. Nutrient uptake and alkaline phosphatase (EC 3:1:3:1) activity of *Emiliania huxleyii* (Prymnesiophyceae) during growth under N and P limitation in continuous cultures. J. Phycol., 36: 87-96.

26.Graziano, L.M., Roche, J.L. and Geider, R.J. 1996. Physiological responses to phosphorus limitation in batch and steady state cultures of *Dunaliella tertiolecta* (Chlorophyta): A unique stress protein as an indicator of phosphate deficiency. J. Phycol., 32: 825-838.

27.Fuhs, W.G., Demmerle, S.D., Canelli, E. and Chen, M. 1972. Characterization of phosphorus-limited plankton algae (with reflections on the limiting nutrient concept). In Likens, G. (Ed.) Limnol. Oceanogr. Spec. Symp., 1: p.113-133.

28.Perry, M.J. 1976. Phosphorus utilization by an oceanic diatom in phosphorus limited chemostat culture and in the oligotrophic waters of the central North Pacific. Limnol. Oceanogr., 21: 88-107.

29.Nalewajko, C. and Lean, D.R.S. 1978. Phosphorus kinetics-algal growth relationships in batch cultures. Mitt. Int. Verein. Limnol., 21: 184-192.

30.Burmaster, D.E. and Chisholm, S.W. 1979. A comparison of two methods for measuring phosphate uptake by *Monochrysis lutheri* Droop grown in continuous culture. J.Exp.Mar.Biol. Ecol., 39: 187-202.

31.Thomas, C.R. 1979. Olisthodiscus luteus. III. Uptake and utilization of nitrogen and phosphorus. J. Phycol., 15: 5-12.

32.Rao, V.N.R. and Selvarani, V. 1989. Phosphate uptake in Amphora coffeaeformis (Agardh) Kütz, Navicula pelliculosa (Breb.) Hilse and Thalassiosira fluviatilis Husteadt. Phykos, 28(1&2): 216-230.

33.Lubian, L.M., Blasco, J. and Establier, R. 1992. A comparitive study of acid and alkaline phosphatase activity in several strains of *Nannochloris* (Chlorophyceae) and *Nannochloropsis* (Eustigmatophyceae). Br. Phycol. J., 27: 119-130.