Regulation of alkaline phosphatase synthesis in *Phaeocystis* sp.

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ABSTRACT: In short-term experiments, cells of *Phaeocystis* sp. grown under phosphate-sufficient conditions were harvested and transferred to phosphate-depleted medium. In these cultures alkaline phosphatase activity (APA) developed after a lag-phase of 8 h; addition of cycloheximide ($10~\mu mol~l^{-1}$) partly inhibited APA development. Addition of organic phosphate (AMP, $0.5~\mu mol~l^{-1}$) did not have an inducing effect on alkaline phosphatase synthesis. These results indicate that in *Phaeocystis* sp. alkaline phosphatase synthesis is controlled by the external phosphate concentration. Synthesis is derepressed at low inorganic phosphate concentration in the medium; the threshold concentration for derepression was ca $0.5~\mu mol~l^{-1}$ phosphate. The dark synthesis rate of alkaline phosphatase in *Phaeocystis* sp. was not correlated with the length of the preceding light period for both colony cells and single cells, although at least a 2 h light period was needed by colony cells to give an enhanced dark synthesis rate compared with a dark control. In a batch culture growth experiment APA developed during the exponential growth phase of *Phaeocystis* sp. APA development was found to be independent of the initial N/P ratio of the medium. The results imply that APA is not a good indicator of phosphorus limitation in *Phaeocystis* sp.

INTRODUCTION

Cell-surface phosphatases are enzymes that are associated with freshwater and marine planktonic algae (Kuenzler & Perras 1965, Healey 1973) and bacteria (Kobori & Taga 1979). Their function is the release of inorganic phosphate (Pi) from organic phosphates that are suspended in the water through hydrolysis of the P-O-C bond (Cembella et al. 1983). Normally, cell-surface phosphatases of unicellular algae have alkaline pH optima (Kuenzler & Perras 1965) although examples of acid extracellular phosphatases are known (Price 1962, Patni & Aaronson 1977). There is some controversy concerning the regulation of phosphatase synthesis in unicellular algae (review by Cembella et al. 1983). In some cases algal phosphorus content is found to be the factor controlling phosphatase synthesis (Møller et al. 1975), while in other algae low external phosphate concentrations seem to initiate synthesis (Lien & Knutsen 1973, Rivkin & Swift 1980). An inducing effect of dissolved organic phosphates on phosphatase synthesis has been observed as well (Guerrini et al. 1971, Cembella et al. 1984). It is clear that knowledge of the factors controlling alkaline phosphatase synthesis in the dominating algae is necessary

for a correct interpretation of alkaline phosphatase activity (APA) data from field situations. Nevertheless, the occurrence of APA in natural systems is often used as an indicator of phosphate limitation in planktonic algae (e.g. Solórzano 1978, Smith & Kalff 1981, Vincent 1981).

The marine colony-forming flagellate *Phaeocystis* sp. (Prymnesiophyceae) also produces an alkaline phosphatase (Veldhuis & Admiraal 1987). During *Phaeocystis* sp. blooms in the Southern Bight of the North Sea, high APA values were measured when inorganic phosphate concentrations in the water were low (Veldhuis et al. 1987). Because ambient N/P ratios were high, these observations suggest a P-deficiency of the *Phaeocystis* sp. population. In order to be able to decide whether or not APA is a parameter indicative of P-deficiency in *Phaeocystis* sp., some factors controlling alkaline phosphatase activity in *Phaeocystis* sp. were examined.

MATERIAL AND METHODS

Algal strain. *Phaeocystis* sp. was isolated from the Southern Bight of the North Sea. This strain was identified as *Phaeocystis pouchetii* by Veldhuis & Admiraal

(1987) but in accordance with Sournia (1988) we will refer to it as *Phaeocystis* sp. or simply *Phaeocystis* until the taxonomic problems concerning this genus are solved.

Culturing conditions. *Phaeocystis* sp. was cultured uni-algal but not axenic in the medium described by Veldhuis & Admiraal (1987). In this medium NH_4Cl was omitted so nitrate was the only nitrogen source. *Phaeocystis* cultures were incubated in serum bottles placed on a rolling device (3 rpm) and illuminated by cool-white fluorescent tubes (Philips no. 34; light intensity 115 $\mu E\ m^{-2}\ s^{-1}$) in a 14 h light:10 h dark cycle at 11 °C.

Short-term experiments. The regulation of alkaline phosphatase synthesis in Pi-sufficient *Phaeocystis* cells was examined in short-term experiments (Table 1, Expts 1, 3 and 4). The role of external phosphate concentration and of light availability in the regulating process received special emphasis. In preparation of these experiments *Phaeocystis* was precultured in 3 l serum bottles in medium with initial nitrate and phosphate concentrations of 150 and 5 $\mu mol\ l^{-1}$, respectively (Pi-sufficient cultures). These cultures were harvested during the exponential growth phase when *Phaeocystis* cell numbers were 50 to $100 \times 10^6\ l^{-1}$, nitrate concentration was ± 100 $\mu mol\ l^{-1}$ and phosphate concentration was still more than 2 $\mu mol\ l^{-1}$. Harvesting (see Fig. 1) of *Phaeocystis* colonies was done by filtering the culture carefully through 50 μm mesh size netting, by which most colonies (and a small part of the single cells) were retained. In Expt 4, to obtain pure colonies, the filtering was repeated twice to remove all single cells. In this experiment single cells of *Phaeocystis* were harvested from a second Pi-sufficient culture by centrifugation (1 min, 1000 × *g*) after filtration of the culture through a 20 μm mesh size netting to

remove all colonies. After centrifugation, pellets containing single cells only were resuspended in fresh medium. In all short-term experiments harvested cells were transferred to 3 l fresh medium, containing 100 $\mu mol\ l^{-1}$ nitrate but no phosphate. After transfer the phosphate concentration in the new cultures was always less than 0.15 $\mu mol\ l^{-1}$ and cell number was 10 to $50 \times 10^6\ l^{-1}$ (Pi-depleted cultures). Microscopic examination of colonies and single cells showed that transferring caused no damage. Transfer from Pi-sufficient to Pi-depleted medium was completed within 3 h after the start of the light period in Expts 1 and 3 and before the end of the dark period in Expt 4. Before use in a short-term experiment the 3 l Pi-depleted cultures were divided into 4 to 12 subcultures that were treated in different ways (see Table 1). The subcultures were incubated in serum bottles under the same conditions as the precultures and the development of alkaline phosphatase activity with time was followed. At the start and end of each experiment samples were taken for *Phaeocystis* and bacterial cell counts and for nutrient analysis.

Where indicated cycloheximide was added to subcultures from a 1 mmol l^{-1} stock solution. Cycloheximide is a competitive inhibitor of protein synthesis in eukaryotic organisms (Sisler & Siegel 1967). This means that the extent of protein synthesis inhibition depends on the cycloheximide concentration. Based on literature references (McMahon 1975, Aaronson & Patni 1978) we used a 10 $\mu mol\ l^{-1}$ cycloheximide concentration in Expt 1. Comparison of APA development in cycloheximide treated subcultures with control subcultures that contained bacteria only (obtained by filtering an identical subculture through Whatman GF/C glass-fiber filter) indicated that a concentration of 25 $\mu mol\ l^{-1}$ cycloheximide inhibits alkaline phosphatase

Table 1. Manipulation of subcultures in experiments. Subcultures in Expts 1 to 3 contained both colonies and single cells of *Phaeocystis*. Subcultures in Expt 4 contained colonies or single cells. Cont. = continuous

Expt 1 Derepression of alkaline phosphatase

Subculture:	1	2	3	4
Addition:	–	10 µM cycloheximide	0.5 µM AMP	Pi

Expt 2. Relation between N/P ratio and APA development in batch cultures

Subculture:	1	2	3
N/P ratio:	7.6	13.1	19.1

Expt 3. Relation between external inorganic phosphate (Pi) concentration and APA development in algae and bacteria

Subculture:	1	2	3	4	5	6	7	8	9	10	11	12
Pi (µM):	0.17	0.32	0.46	0.51	0.62	1.11	0.18	0.30	0.37	0.53	0.64	1.0
Cycloheximide (µM):	0	0	0	0	0	0	25	25	25	25	25	25

Expt 4. Relation between lightperiod (h) and APA development in colonies and single cells

	Colonies						Single cells			
Subculture:	1	2	3	4	5	6	1	2	3	4
Lightperiod:	0	1	2	5	8	Cont.	0	5	8	Cont.

HARVESTING PROCEDURE FOR:

COLONY CELLS   SINGLE CELLS

preculture   preculture
colonies and   colonies and
single cells   single cells

colonies   50 um filter   20 um filter
                                        (colonies are retained)

                                        single cells
                                        1 min, 1000 rpm
fresh medium   single cells

                                        pellet

                                        fresh medium

subcultures   subcultures

Fig. 1. *Phaeocystis* sp. Schematic representation of harvesting procedure for both colonies and single cells. For further explanation see text

synthesis in *Phaeocystis* completely but a cyclohex-imide concentration of 10 μmol l^{-1} does not. Therefore, 25 μmol l^{-1} cycloheximide was added to subcultures in Expt 3.

In Expt 1 0.5 μmol l^{-1} AMP was added to a subculture from a 1 mmol l^{-1} stock solution. AMP is a substrate for the alkaline phosphatase enzyme of *Phaeocystis* (Admiraal & Veldhuis 1987). In Expts 1 and 3 inorganic phosphate was added from a 2 mmol l^{-1} stock solution. All stock solutions were made with double distilled water. In Expt 4 where the influence of different light periods on APA development was examined subcultures were excluded from light by wrapping the bottles in aluminum foil.

Batch culture experiment. The relation between initial N/P ratio in the medium and APA development in *Phaeocystis* was studied in a batch culture experiment (Expt 2 in Table 1). *Phaeocystis* was grown in three 1 l serum bottles in medium with fixed initial phosphate concentration (ca 2.5 μmol l^{-1}) but with different initial N/P ratio (7.6, 13.1, and 19.1) in each bottle. Culture conditions were as described above and samples for nutrient analysis, cell counts and APA measurement were taken from each culture at regular intervals.

Biological and chemical analysis. *Phaeocystis* cells were counted by the Utermöhl sedimentation techni-

que (Utermöhl 1958) after fixation of samples with buffered Lugol solution. Single cells and colony cells were counted separately. Bacterial cell counts were done by fluorescence microscopy after fixation of samples with 4 % formalin and staining with DAPI (diamidinopherylindole). Protein was measured according to Lowry et al. (1951). Nitrate concentration was determined with an auto-analyzer according to Strickland & Parsons (1972), inorganic phosphate concentration following Murphy & Riley (1962) and cellular phosphorus concentration as inorganic phosphate after persulfate oxidation (1 h at 120 °C) of pellets. APA was measured fluorimetrically using 3-o-methyl-fluorescein phosphate (MFP) as substrate (Perry 1972). For most experiments APA is expressed per 10^6 *Phaeocystis* cells. In these cases APA values were corrected for changes in cell number during the experiment. Bacterial contribution to culture APA was determined in subcultures that received 25 μmol l^{-1} cycloheximide to inhibit algal APA development, or in the filtrates of samples filtered over (Whatman GF/C) glass-fiber filters taken from subcultures. In the latter case bacterial cells were counted in filtrate and subculture in order to compensate for filtration losses of bacteria. Free APA (the APA fraction passing a 0.2 μm filter) was never found in the cultures.

RESULTS

Experiment 1

A Pi-sufficient culture of *Phaeocystis* was transferred to Pi-depleted medium and divided into 4 subcultures (Table 1). At that moment the phosphorus content of the cells in the subcultures was 0.041 pmol cell^{-1}. One subculture received no further additions and here APA developed after a lag-phase of ca 8 h (Fig. 2). APA increased linearly in time over the next 14 h. Addition of cycloheximide (10 μmol l^{-1}) to a subculture at the start of the experiment extended the lag phase to ca 12 h and the following increase in APA with time was much slower. Addition of AMP to a subculture had no effect on the lag phase but the APA developed poorly. In the control (Pi-sufficient) subculture no APA developed during the experiment.

Experiment 2

In 3 batch cultures of *Phaeocystis* with the same initial phosphate concentration but with different initial molar N/P ratios (7.6, 13.1, and 19.1, respectively) APA developed as the phosphate concentration in the medium fell below 0.5 μmol l^{-1} (Fig. 3). At that moment the cells were still in the exponential growth phase and

the cellular phosphorus content was > 0.06 pmol cell^{-1}. In the stationary phase of P-limited batch cultures the minimal phosphorus content of our *Phaeocystis* strain was found to be 0.01 pmol cell^{-1} (results not shown). The *Phaeocystis* cells in the cultures of Expt 2 were therefore clearly not phosphorus limited as APA developed. Bacterial contribution to APA was not significant in the batch cultures.

Experiment 3

The role of external phosphate concentration in APA development was further investigated during another short-term experiment. A Pi-sufficient culture of *Phaeocystis* sp. (cellular phosphorus content 0.025 pmol cell^{-1}) was harvested and transferred to Pi-free medium. This culture was then divided over 12 serum bottles to which different amounts of phosphate stock solution were added (final concentration 0.17 to 1.11 μmol l^{-1}). To 6 bottles cycloheximide was added (final concentration 25 μmol l^{-1}) to determine bacterial APA (Table 1). After 24 h the APA in all bottles was measured in duplicate. *Phaeocystis* APA in the subcultures without cycloheximide was determined by subtracting bacterial APA from total APA. In Fig. 4 the relation is shown between the initial phosphate concentration in the medium and the APA after 24 h for both bacteria and *Phaeocystis* cells.
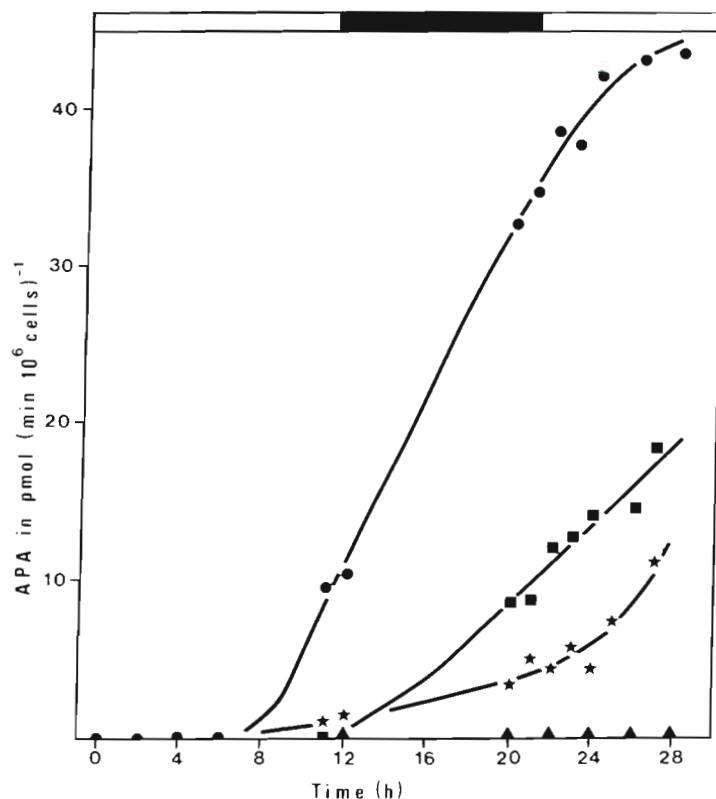

Fig. 2. *Phaeocystis* sp. Derepression of alkaline phosphatase in subcultures after transfer from Pi-sufficient to Pi-depleted medium at t = 0. Subcultures contained 35 % colony cells. Bar at top of figure indicates dark period. Curves are: (●) Pi-depleted medium, [PO₄] < 0.1 μM at t = 0; (■) Pi-depleted medium, 10 μM cycloheximide added at t = 0; (★) Pi-depleted medium, 0.5 μM AMP added at t = 0; (▲) Pi-sufficient medium, 8.0 μM PO₄ added at t = 0
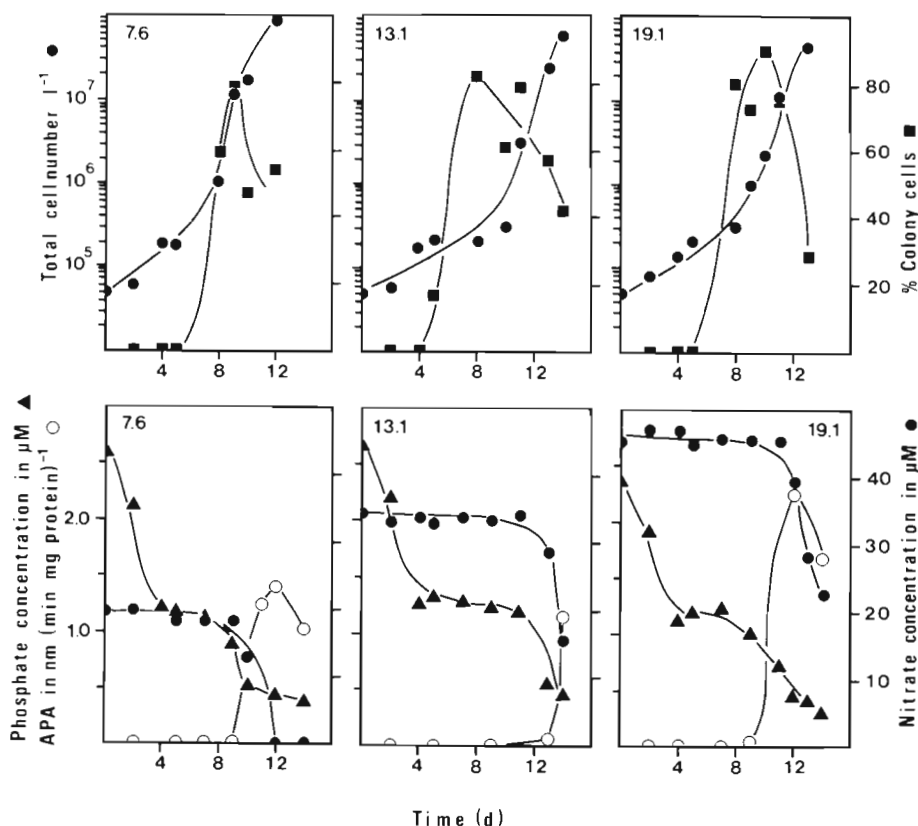
Fig. 3. *Phaeocystis* sp. Total cell number, percentage colony cells, nitrate and phosphate concentrations and alkaline phosphatase activity (APA) in 3 batch cultures with different initial molar N/P ratio (indicated in upper corner of each figure)

Apparently, a threshold concentration of phosphate in the medium existed below which alkaline phosphatase synthesis was derepressed. The value of this threshold concentration could only be estimated because the phosphate concentration in the medium was not kept at a constant level during the experiment. The value lies below 0.5 µmol l^{-1} inorganic phosphate for both *Phaeocystis* cells and bacteria.

Experiment 4

In order to investigate the influence of light on APA development, Pi-depleted subcultures containing either colony cells or single cells of *Phaeocystis* were exposed to light for different periods (Table 1). The cell types were harvested separately from different Pi-sufficient cultures. Harvesting (t = 0) was done at the end of the 10 h dark period so the energy reserves of the cells were low, but probably not depleted. The bacterial contribution to subculture APA was determined at the end of the experiment. The results of this experiment (Fig. 5) show that a 5 h light period was sufficient for both colony cells and single cells of *Phaeocystis* to continue alkaline phosphatase synthesis in the dark at the same rate as in the light. For colony cells a 1 or 2 h light period did not result in a higher alkaline phos-

phatase synthesis rate compared with the dark control. At the end of the experiment, bacterial APA values were ca 60 % and 45 % of total APA in the dark bottle for subcultures of colony cells and single cells, respectively. Values were comparable between the subcultures of each cell type.

DISCUSSION

The results shown in Figs. 2 to 4 clearly demonstrate that alkaline phosphatase synthesis in *Phaeocystis* is controlled by the external phosphate concentration. At the moment of harvesting the phosphorus content of *Phaeocystis* cells was 0.02 to 0.06 pmol $cell^{-1}$. The minimal phosphorus content of 0.01 pmol l^{-1} found for our *Phaeocystis* strain is in close agreement with results of Jahnke (1989) who found a minimal content of 0.008 pmole $cell^{-1}$ in *Phaeocystis* strains isolated from the southern North Sea. Even when growing at μ_{max} ($\approx 0.8\,d^{-1}$ in our experiments), the lag-period of 8 h would have caused only a 23 % reduction of the internal phosphorus content in *Phaeocystis* cells in experiments shown in Figs. 2 and 4. Internal P was therefore not limiting at the moment APA development started. The results of the batch culture growth experiment (Fig. 3) support this conclusion since APA development
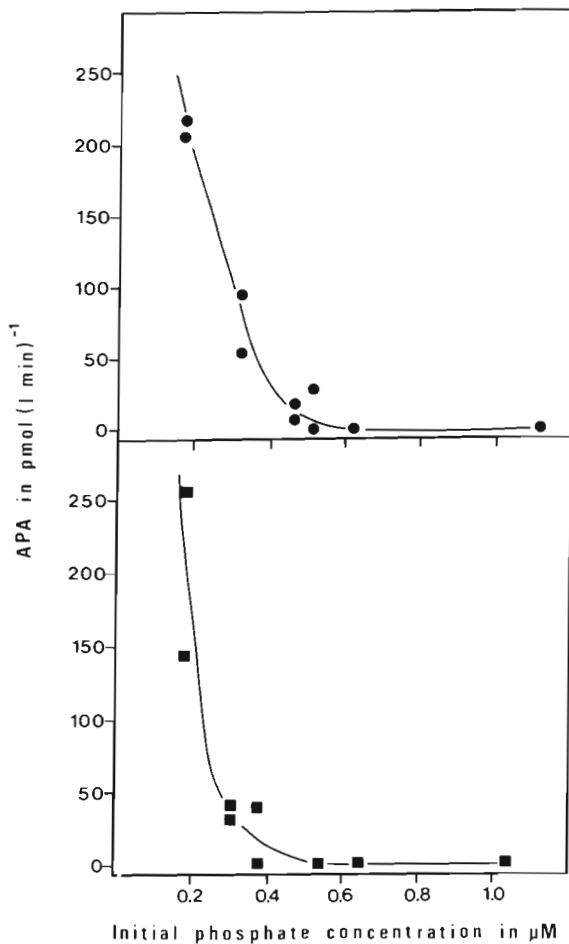
Fig. 4. Influence of phosphate concentration on the development of APA in cells of *Phaeocystis* sp. and in bacteria. Bacterial APA (■) was measured after 24 h in cultures that received 25 μmol l^{-1} cycloheximide at the start of the experiment. Algal APA (●) was calculated by substracting bacterial APA from total culture APA

was initiated during the exponential (not-limited) growth phase. Other authors also have found evidence that the external phosphate concentration regulates phosphatase synthesis in algae. Lien & Knutsen (1973) found an acid phosphatase in *Chlamydomonas reinhardti* that was derepressed after a lag-phase of 2 h upon transfer of the algae to Pi-depleted medium. Addition of inorganic phosphate to this culture stopped acid phosphatase synthesis. For a freshwater lake Chróst et al. (1989) found that algal APA development was initiated by the decrease of inorganic phosphate in the water. Taft et al. (1977) concluded the same for a plankton assemblage in a saltwater bay.

Perry (1976) measured low APA in a N-limited chemostat culture of *Thalassiosira pseudonana* growing at low μ and a residual phosphate concentration of 0.3 μmol l^{-1} According to Perry this low APA was

caused by leakage of the cells due to the low growth rate. In view of our results (Figs. 2 to 4) it can be suggested that the APA of *T pseudonana* was caused by the low phosphate concentration in the medium even though N was the limiting factor.

In our batch culture experiment (Fig. 3) APA development showed no relation with initial N/P-ratio in the medium. APA developed in all cultures when the external inorganic phosphate concentration fell below 0.5 μmol l^{-1}. It would be tempting to state that in the subculture with initial N/P ratio of 7.6 nitrogen was limiting growth since all nitrate in the medium was consumed. This would mean that APA occurred in *Phaeocystis* under N-limited conditions. However, in all batch cultures the nitrate concentration showed only a small decrease during the first part of the exponential growth phase and a steep fall during the second part. The amount of nitrate consumed in the early exponential growth phase was insufficient to account for the concomitant increase in *Phaeocystis* cell number. This might indicate that an internal store of nitrogen in the *Phaeocystis* cells is used during the early exponential growth phase. If *Phaeocystis* is indeed able to store nitrogen then N-limitation cannot be predicted from nitrate depletion in the medium. The results of the batch culture experiment, therefore, only show that *Phaeocystis* developed high APA under P-sufficient conditions.

Veldhuis et al. (1987) found threshold phosphate concentrations of 0.5 and 0.2 μmol l^{-1} for APA development in batch cultures and natural populations of *Phaeocystis*, respectively. Their results are in agreement with the Pi-APA relation found here (Fig. 4). The lower threshold concentration for natural populations may reflect a different physiological state of the cells, caused by different growth conditions.

The concentration of 10 μmol l^{-1} cycloheximide used in Expt 1 (Fig. 2) was not sufficient for a complete inhibition of protein synthesis in *Phaeocystis*. In the subculture with cycloheximide the APA originated, therefore, from both bacteria and *Phaeocystis* cells. In this subculture the lag-phase before APA development was prolonged. Since cycloheximide inhibits all protein synthesis and to some extent other cell processes too (McMahon 1975) the longer lag-phase may be caused by a general inhibition of cell metabolism in the *Phaeocystis* cells. Also, this result indicates that the lag-phase for APA development in the contaminating bacteria is longer than in *Phaeocystis*. In the cycloheximide treated subculture the rate of APA development was slow compared with the subculture without additions since *Phaeocystis* contributed only partly in alkaline phosphatase synthesis. These differences between APA development in the subculture without additions and the subculture with added cycloheximide clearly
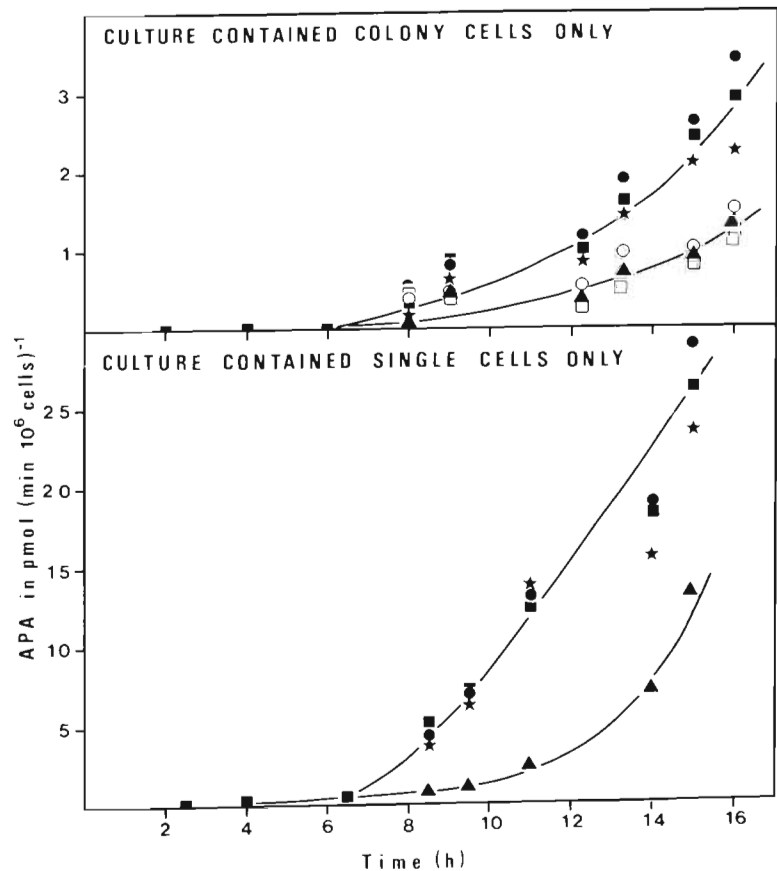
Fig. 5. *Phaeocystis* sp. Development of APA in subcultures in relation to light conditions; differences between colony cells and single cells. Cultures were harvested at the end of a 10 h dark period (t = 0). Subcultures were illuminated for the following periods after t = 0: 0 h (▲), 1 h (○), 2 h (□), 5 h (★), 8 h (●), and in continuous (■) light, Note different scales on y-axis

indicate that upon transfer of *Phaeocystis* cells to Pi-depleted medium de novo protein synthesis was required for the development of APA. In other algae alkaline phosphatase is also not constitutive (Kuenzler & Perras 1965, Cembella et al. 1983).

The presence of 0.5 μmol l^{-1} AMP did not stimulate alkaline phosphatase synthesis in *Phaeocystis* (Fig. 2). Instead APA development in the subculture with AMP was slow compared with that without additions. Partly this was caused by the fact that AMP is a competitive inhibitor of the hydrolysis of MFP by alkaline phosphatase. Inorganic phosphate in the medium has the same effect (Pettersson 1979). The true APA that developed in the cultures during the experiments was therefore higher than the measured APA. The concentrations of AMP and inorganic phosphate were not followed with time but the effect of competitive inhibition on APA values was calculated by the use of the inhibition constants of AMP and Pi (0.46 and 1.18 μM, respectively; preliminary results). The true APA in the subculture with AMP (Fig. 2) would in the most extreme case (no AMP hydrolysed during the experiment) be 2 times the measured APA, while the low Pi concentrations in the subcultures had no significant influence on the APA values. Taking this into account

the APA development in the subculture with AMP was still slow compared with the control. It cannot be excluded though that AMP has a stronger competitive effect on the reaction of bacterial alkaline phosphatase with MFP thereby causing an underestimation of the calculated APA values. Since it is known that bacteria can assimilate organic phosphates (Fenchel & Blackburn 1979) the difference might also be explained by assuming that bacterial APA development in the subculture with AMP was less as a consequence of the direct uptake of AMP from the medium by the bacteria.

The energy and organic carbon required by *Phaeocystis* for alkaline phosphatase synthesis (as for protein synthesis in general) originates from photosynthesis in the light and from photosynthetically fixed C-reserve material in the dark. Lancelot et al. (1986) found a dependence on 'previous light history' of dark protein synthesis in *Phaeocystis* colonies. A light period of at least 3 h was needed by *Phaeocystis* to allow continued protein synthesis in the following dark period. Above this time threshold the protein synthesis rate in the dark was positively correlated with the length of the preceding light period. In our Expt 4 a 2 h light period did not enhance the alkaline phosphatase synthesis rate in colonies of *Phaeocystis* compared with

the dark control (Fig. 5). A light period of 5 h or more did give an enhanced dark alkaline phosphatase synthesis rate for both colonies and single cells of *Phaeocystis* but there was no positive correlation between synthesis rate and length of the light period as was found by Lancelot et al. (1986). Apparently, after a light period of 5 h or more energy supply was not limiting in *Phaeocystis* and the alkaline phosphatase synthesis rate was determined by the external phosphate concentration which was the same in all subcultures. In the experiment shown in Fig. 5, single cells expressed a higher APA compared with colony cells. This is probably caused by a difference in the growth phase of the two cultures at the moment they were harvested.

Alkaline phosphatase synthesis in bacteria contaminating the algal cultures appears to be regulated in the same way as in *Phaeocystis* (Fig. 4). Production of Pi-repressible alkaline phosphatases is known in several bacteria (Torriani 1960, Day & Ingram 1973, Hassan & Pratt 1977), but other bacteria have constitutive alkaline phosphatases (Hassan & Pratt 1977). Selection in the bacterial population of the *Phaeocystis* cultures may have favoured a few bacterial species. The results therefore, only implicate the fact that one or more marine bacteria have a Pi-repressible alkaline phosphatase.

The presence of APA in natural waters is often regarded as an indicator of P-limitation (e.g. Perry 1972, Chiaudani & Vighi 1982, Myklestad & Sakshaug 1983). Our results show that the presence of APA during *Phaeocystis* blooms only indicates a low external phosphate concentration and does not need to imply P-limitation.

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