An examination of the factors involved in determining phosphatase activities in estuarine water. 1: Analytical procedures

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Abstract

The effects of assay pH, magnesium addition, temperature, sodium azide, and of sample storage and filtration on the apparent phosphatase activities in natural water bodies, and in particular estuaries, have been examined. Phosphatase activity was assessed by monitoring the cleavage of *p*-nitrophenol from *p*-nitrophenyl phosphate (*p*NPP).

Magnesium concentrations in natural waters were found to be generally in excess of the requirement of the assay procedure. Several pH maxima occurred for phosphatase activities in the estuarine waters examined. These optima were not constant with respect to sampling stations or sampling time. This was considered to be, in part, the result of differences in the biomass composition. Sample storage times and temperatures employed in the present study did not influence the phosphatase activities of these low biomass waters. Phosphatase activities increased with increased incubation temperature. The rate of orthophosphate release from *p*NPP during incubation was linear over time to 120 h. Sodium azide was found to be an adequate preservative when long incubation times were required.

The effects of assay conditions are discussed in relation to the assay procedures and data interpretation.

Introduction

When in the organic form, phosphorus is not directly utilizable by most organisms. Phosphatases hydrolyze the ester linkages of organic phosphorus molecules, making orthophosphate available for uptake and metabolism. Many organisms will synthesize phosphatases in response to phosphorus deficient conditions. Conversely, in many organisms, synthesis of phosphatase may be repressed by excess available orthophosphate (cf. Aaronson *et al.*, 1976). Therefore, it has been suggested that the phosphatase activity in a body of water can be used as an index of the phosphorus nutritional status of the system (cf. Berman, 1970; Jones, 1972; Perry, 1972). In order to be meaningful as an index, adequately defined assay procedures must be followed.

The most commonly used procedures for assaying phosphatase activity are based on monitoring cleavage of artificial substrates, either *p*-nitrophenyl phosphate (Reichardt *et al.*, 1967) or 3-o-methyl fluorescein phosphate (Perry, 1972). Use of the latter substrate is more sensitive.

Problems with assessing phosphatase activities in natural waters can be grouped into two categories. The first is related to the details of the assay method and sample treatment, while the second deals with adequate sampling procedures. Meaningful comparisons within and between studies can be made only when both are adequately defined. In this paper, the variations in apparent phosphatase activities due to assay conditions (pH, cation addition, incubation time and temperature, preservative addition, sample filtration, and sample storage time and temperature) are examined.

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Materials and methods

1. Routine phosphatase assay

Phosphatase activities were measured using essentially the method of Reichardt *et al.* (1967).

The reaction mixture was 2:2:1 (v/v/v) of water sample, buffer, and substrate. The buffer systems used were 0.1 M Tris-HCl, pH 7.5 or 8.6, for neutral or alkaline phosphatases, or 1 M citric acid-sodium citrate buffer, pH 5.6, for acid phosphatases. The substrate was 0.7 mM *p*-nitrophenyl phosphate (*p*NPP, Sigma 104) in a 10^{-2} M $MgSO_4$ solution. Incubation was at 25 °C in the dark for 24 or 48 h. Sodium azide (5 mM) was added as a preservative to inhibit microbial growth and to preclude phosphatase synthesis. Substrate and sample controls were included in all experiments. The enzyme activity was measured on a Varian Techtron (Model 635) spectrophotometer at 410 nm. For alkaline and neutral phosphatases this was done directly on the samples, but for acid phosphatases the colour of the reaction mixture was developed by adding an equal volume of 0.1 M NaOH. This solution was made up just prior to the use with high purity NaOH, since carbonates which formed in the NaOH solution caused precipitates in the reaction mixture. Measurements were corrected for sample and substrate controls. Results are generally reported in terms of absorbance units at 410 nm in a 1 cm quartz cuvette.

Error bars have not been included in Figs. 8, 9 & 10, but the variances in the assay method are ± 11.4% after 24 h sample incubation, and ± 5.7% after 48 h incubation. These are based on the March 1978 grid study data (see accompanying paper, Huber & Kidby). Variances were calculated using:

$$S^2 = \frac{1}{2n} \sum_{i=1}^{n} (\log x_{i1} - \log x_{i2})^2$$

where i = the sampling site (n = 36) and x_{i1} and x_{i2} are replicate assays of a sample. Sample variation within one sampling site is discussed in the accompanying paper (Huber & Kidby).

Details of variations of the basic methods are given in Results and Discussion.

2. Standard phosphatase assays

Sigma alkaline phosphatase (calf intestinal mucosa, 1.2 activity units mg^{-1}; 1 unit converts 1 μmol *p*NPP min^{-1} at 37 °C, pH 9–10) was used. The standard solution was $8.28 \cdot 10^{-3}$ activity units per 5 ml total volume. The time response was examined over a 3 h period.

3. Sampling stations

All samples were taken from the Peel-Harvey Estuary located in the south-west of Western Australia. The locations of the sampling sites are shown in Fig. 1.

Results and discussion

1. Standard phosphatase assay

The results of a standard phosphatase assay (bo-

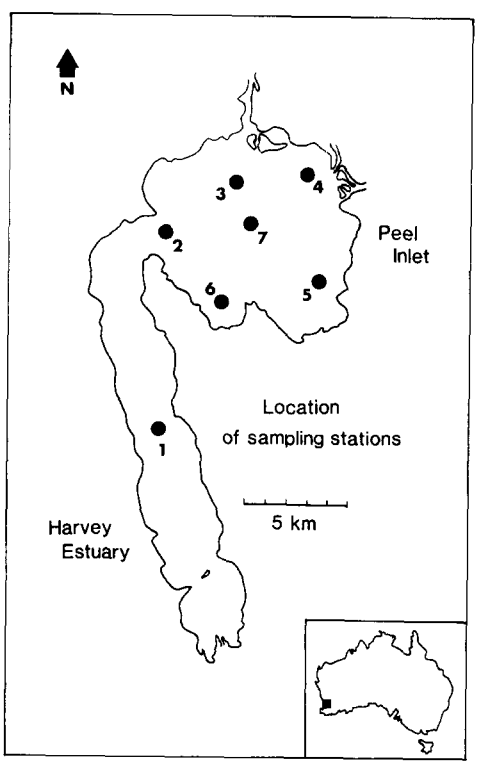

Fig. 1. Location of sampling stations.

vine phosphatase, pH 8.6) are presented in Fig. 2. The equation describing the time response curve was: Absorbance (410 nm) = $4.16 \cdot 10^{-3}$(time in minutes) × 0.03, $r^2 = 0.996$.

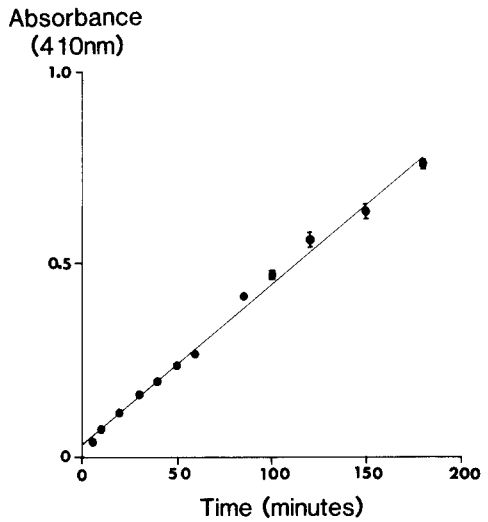

Fig. 2. Timecourse of the release of *p*-nitrophenol (*p*-NP) (absorbance at 410 nm) from *p*-nitrophenyl phosphate (*p*-NPP) by phosphatase (calf mucosal).

2. *Assay conditions*

The assay conditions used in many of the more recent studies on phosphatase are summarized in Table 1. The parameters listed in the table are discussed below.

(a) Cation requirement. The final magnesium concentration used in routine analyses was 2 mM $MgSO_4$. To test for a requirement, this concentration was compared to no added magnesium in two surface water samples from sampling stations 1 and 6. The results are presented in Fig. 3; no significant differences were found.

Table 1. Assay conditions used in recent phosphatase studies.

Author	Phosphatase source	Substrate	Cation addition	pH	Buffer	Incubation temp. °C	Incubation time	Preservative	Test wavelength
1	lake water	*p*-NPP	$MgCl_2$	8.4	Tris-HCl	25	96 h	chloroform	418 nm
1	lake water	'natural'	$MgCl_2$	8.4	Tris-HCl	25	96 h	chloroform	418 nm
2	*Anabaena*	*p*-NPP	$MgCl_2$	8.5	Tris-HCl	30	30 min	none	410 nm
3	algal spp.	*p*-NPP	$MgCl_2$	8.5,9.0	Tris	35–37	0.25–2 h	none	395 nm
4	bacterial	*p*-NPP	–	3.0–6.5	Clark-Lub's	0–70	0–60 min	none	400 nm
4	cultures	*p*-NPP	–	6.5–9.5	Michael's	0–70	0–60 min	none	400 nm
4	cultures	*p*-NPP	–	9.5–12.0	Sorensen's	0–70	0–60 min	none	400 nm
4	natural water	*p*-NPP	–	-	none	15–25	10 h	none	400 nm
5	algal spp.	MFP[a]	Ca, Mg	8.5	Tris	35	0.5–1.5 h	none	fluorometer
6	periphyton	*p*-NPP	$MgCl_2$	8.5	Tris	35–37	2 h	-	410 nm
7	sea urchin	*p*-NPP	none	10.5	glycine	38	various	none	410 nm
8	plankton	MFP[a]	none	6.5	Tris-HCl	20	–	-	418 nm
9	lake water	*p*-NPP	none	7.6	Tris	15	3 days	chloroform	418 nm
10	marine algae	*p*-NPP	$MgCl_2$	3–10	citrate, Tris	–	-	none	410 nm
11	*Cladophora*	PP & TPP[b]	$MgCl_2$	7.8	Tris	30	various	none	410 nm
12	*Ochromonas*	*p*-NPP and	several	3.6	Acetate	30–50	30 min	none	410 nm
	Ochromonas	others	several	8.6	Tris	30–80	10–15 min	none	410 nm
13	seawater	MFP[a]	none	8.5	Tris-HCl	18–23	20–120 min	none	fluorometer
14	lake water	several	none	8.3	Tris	5–20	5–20 min	none	fluorometer
15	lake water	*p*-NPP	none	4.6–9.1	several	25	96 h	-	418 nm
15	cyanophyta	*p*-NPP	none	8.0	Tris-HCl	25	12 h	-	418 nm
16	lake water	*p*-NPP	$MgCl_2$	8.4	Tris-HCl	25	96 h	-	-
17	sediments	*p*-NPP	none	8.6	Tris-HCl	37	60 min	none	418 nm
18	soil	*p*-NPP	$CaCl_2$	6.5	MUB	37	60 min	toluene	400 nm
19	marine algae	*p*-NPP	–	8.6	Tris	25	3–30 h	none	410 nm

[a] 3-o-methylfluorescein phosphate.

[b] Pyrophosphate and tripolyphosphate.

Authors: 1. Berman, 1969; 2. Bone, 1970; 3. Fitzgerald & Nelson, 1966; 4. Hayashi, 1972; 5. Healy & Hendzel, 1979; 6. Hooper-Reid & Robinson, 1978; 7. Hsiao, 1965; 8. Jansson, 1976; 9. Jones, 1972; 10. Kuenzler, 1965; 11. Lin, 1977; 12. Patni *et al.*, 1974; 13. Perry, 1972; 14. Petterson & Jansson, 1978; 15. Reichardt, 1971; 16. Reichardt *et al.*, 1967; 17. Sayler *et al.*, 1979; 18. Tabatabi & Bremner, 1969; 19. Walther & Fries, 1967.

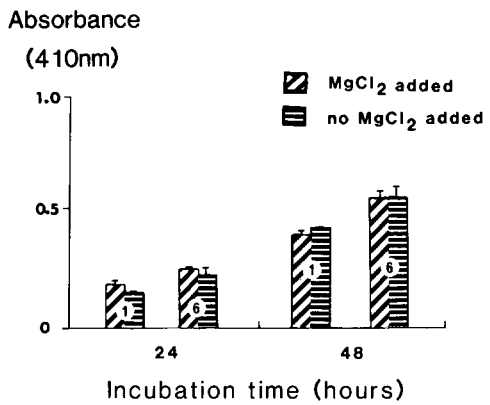

Fig. 3. Effect of added magnesium on phosphatase activity (absorbance at 410 nm) in surface waters from Stations 1 and 6.

Magnesium, at various concentrations, is listed for most phosphatase assay procedures (see Table 1). Lin (1977) found 0.1 M Mg^{2+} to be required by *Cladophora* phosphatases. Patni *et al.* (1974) showed a stimulation by Mg^{2+} of alkaline but not acid phosphatase activity in *Ochromonas*. Healey (1973) showed a minimum requirement for Ca^{2+} of 10 mM which increased when the substrate concentration was lowered. Conversely, Jones (1972) found no significant increase in activity upon addition of Mg^{2+} and concluded that the lake water itself contained ample Mg^{2+}. The methods of Berman (1969), Hayashi (1972) and Tabatabi & Bremner (1969) do not have additional Mg^{2+} salts, but since freshwater, marine and soil samples respectively, were being tested, the sample Mg^{2+} concentration would have been quite high.

The magnesium concentration in Peel-Harvey estuarine waters was found to range from 0.026 to 0.058 M (Huber, 1980). Under the assay conditions used, the sample was diluted to 2/5ths which gave a final concentration of 10 to 23 mM. Therefore five to ten times more magnesium was added by the sample than by the reagent, and no additional magnesium requirement could be shown.

(b) pH profiles. A pH range from 5.0 to 9.5 was examined using citric acid-sodium citrate and TRIS-HCl buffers. The pH intervals examined were 0.5 units except between pH 7.0 to 8.0 where it was necessary to decrease the interval size to 0.2 pH units in order to elucidate the two peaks in that range. Typical results are shown in Figs. 4a–c. In

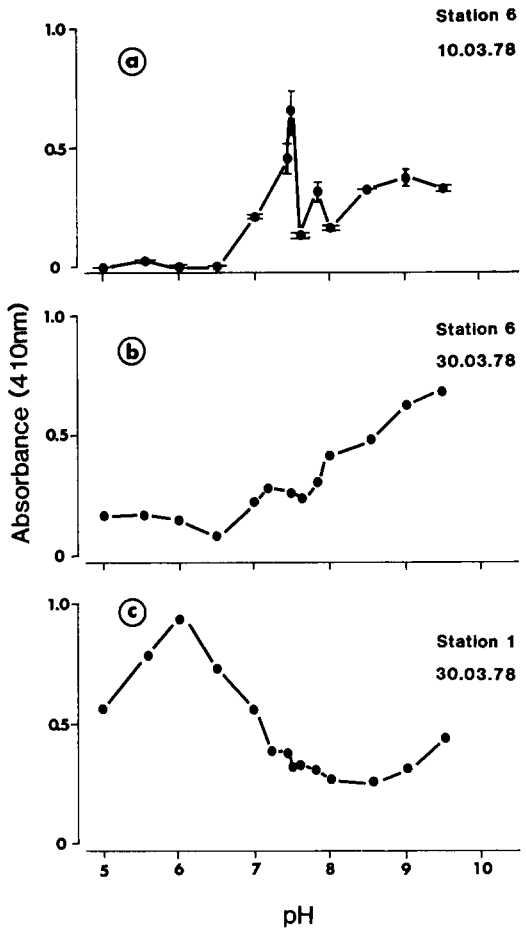

Fig. 4a–c. pH profiles of surface waters from Station 6 surface waters (10.03.78–30.03.78) and Station 1 (30.03.78).

Figure 4a, two sharp peaks are shown at pH 7.5 and 7.8 and a broader peak between pH 8.5 to 9.5; very little acid phosphatase activity was detected. The pH profiles were not uniform between sampling sites or sampling times. For example, at Station 6, the optimum activity was at pH 7.5 on 10.3.78, but at pH 9.5 on 30.3.78 (Figs. 4a, b). On the latter date, the optimum at Station 1 was at pH 6.0 (Fig. 4c).

Variations in pH profiles in natural waters have been described by other researchers. Berman (1970) reported a seasonal change in maximum alkaline phosphatase activity from pH 8.0 to pH 9.6. Reichardt *et al.* (1967) showed maxima at pH 5.6 and pH 8.7–9.1 (0.5 pH unit intervals).

The choice of assay pH can be based upon either the pH optima for the phosphatases of the organism(s) concerned, or the body of water in question.

Table 2. pH optima of phosphatase activities for specific organism.

Organism	Optimum pH	Reference
Aphanizomenon	9.5	Heath & Cooke, 1975
Anabaena	8.3	Healy, 1973
Anabaena	10.0	Healy & Hendzel, 1979
Cryptomonas	8.5	Healy & Hendzel, 1979
Melosira	9.0	Healy & Hendzel, 1979
Scenedesmus	8.0	Healy & Hendzel, 1979
Synanura	5.1	Healy & Hendzel, 1979
Cladophora	7.8	Lin, 1977
16 algal species	4.4-9.8	Kuenzler, 1965
marine bacteria	5.6, 9-10	Hayashi, 1972

However, the range of pH optima for different species is considerable (see Table 2). The choice of the actual water pH enables the measured activities to be more meaningfully related to the prevailing conditions. Taft *et al.* (1977) selected a pH which was within 0.25 units of the water being tested. In the Peel-Harvey Estuary, in the absence of dense blooms, the pH varied between 7.1 to 8.6 (Lukatelich & McComb, 1981). A pH of 7.5 was selected for the routine assay procedure used in this study.

(c) Temperature. Differences in phosphatase activity at 20 ° C and 30 ° C over a pH range of 5.0 to 9.5 were studied for water samples from two sites. The results are presented in Fig. 5. The enzyme activity increased with temperature, but no shift in pH optima occurred. Except for Station 6 water which had very low phosphatase activity at 20 ° C at pH 5.0 and 5.6, the mean increase in activity over the 10 ° C range was 67.9 ± 16.1%.

The optimum temperature for phosphatase activity will depend on the biological composition in the waters sampled. While the activity of phosphatases has been examined at three temperatures, 20 ° C, 25 ° C and 30 ° C, which fall within the ambient range, it should be pointed out that these temperatures will not necessarily be at, or close to, the optima for phosphatases for a number of organisms (see Table 3). In the current work, 25 ° C was chosen for the routine procedure.

(d) Incubation time. Phosphatase activity (pH 7.5) was shown to be linear over time. Linearity was maintained to over 100 h (absorbance range from 0.02 to 1.20).

In most studies, the incubation times used have been less than two or three hours (see Table 1), the time required for significant substrate cleavage being dependent on the enzyme concentration in the system. Berman (1969) found the linear relationship to hold to 120 h in Lake Kinneret waters when chloroform was used to inhibit microbial growth. Reichardt *et al.* (1967) and Jones (1972) measured phosphatase activities in natural waters after incubation for 96 and 72 h, respectively. In the present study, 24 h was selected for routine use because of practicality and inaccuracies inherent in measuring low activity samples tested after short term incubation.

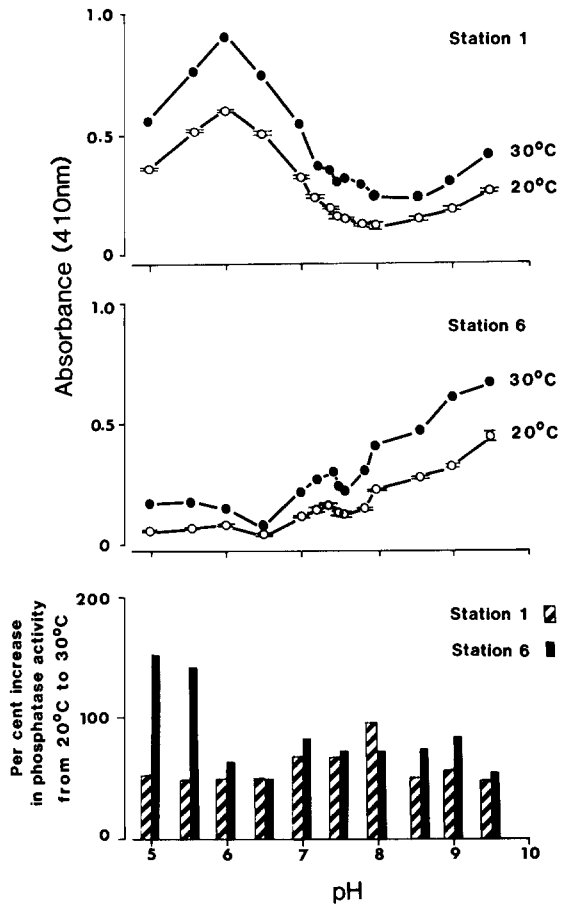

Fig. 5. Temperature-related variations in phosphatase activities (absorbance at 410 nm) of Stations 1 and 6 surface waters at 20 ° C and 30 ° C.

(e) Absorbance wavelength. The wavelengths used to detect *p*-nitrophenol have ranged from 395 to 420 nm; 410 nm is used most commonly (see Table

Table 3. Temperature optima and effects of temperature changes on phosphatase activities.

Enzyme source	Enzyme type	Temperature optimum °C	Q10	Reference
lake waters	mixture	27	–	Reichardt *et al.*, 1967
bacteria - marine	mixture	25–30	–	Hayashi, 1972
bacteria - goldfish ponds	mixture	40–50	–	Hayashi, 1972
Anabaena[a]	mixture	30–40	1.45	Healey, 1973
Chlamydomonas[a]	acid	–	1.8	Lien & Knutsen, 1973
	alkaline	–	2.0	Lien & Knutsen, 1973
Ochromonas[a]	acid	55	–	Patni *et al.*, 1974
	alkaline	37–40	–	Patni *et al.*, 1974
Aphanizomenon[b]	alkaline	40	–	Heath & Cooke, 1975
lake waters	mixture	5–20	1.6	Petterson & Jansson, 1978
natural blooms	mixture	35–40	–	Healy & Hendzel, 1979
Anabaena[a]	mixture	55	–	Healy & Hendzel, 1979
Scenedesmus[a]	mixture	40–45	–	Healy & Hendzel, 1979
Cryptomonas[a]	mixture	35–40	–	Healy & Hendzel, 1979

[a] Laboratory studies.
[b] Laboratory and field studies.

1). This aspect of the assay procedure was not examined and 410 nm was used for all experiments.

3. *Sample treatment*

The following studies were carried out in order to determine which aspects of sample handling and treatment it was necessary to standardize in order to maintain reproducibility of results.

(a) Sample storage. The effects of storage time and temperature were examined in water from two sampling stations. Water samples were taken and held overnight on ice. The following day, an initial phosphatase assay was carried out and subsamples of the water were held at 4 ° or 22 ° C and tested after 24, 48 and 72 h. There appeared to be no significant change in the phosphatase activities over 72 h storage time at either 4 ° C or 22 ° C. Storage conditions for samples are not generally reported, but Walther & Fries (1976) have shown that extracellular alkaline phosphatases (soluble or total) of multicellular marine algae (Rhodophyta and Phaeophyta) are stable at room temperature (25 ° C) and freezing (–5 ° C) for 3 days. However, phosphatase activity was lost on heating (100% loss at 80 ° C for 10 minutes).

In this study, samples were stored on ice or at 4 ° C and analyzed the day after collection.

(b) Sample preservation. With incubation times greater than a few hours, the possibility of increased phosphatase activity due to growing cells must be precluded.

The following preservatives or combinations of preservatives were tested: toluene in ethanol (5% in 0.4%), ethanol (0.4%), sodium azide (5 mM), sodium azide and toluene in ethanol (in above concentrations), sodium azide and chloramphenicol (0.01% each) and azide and chloroform (0.01% and 0.4%, respectively). At 24 h or less, preservative was not required (Table 4). Azide proved to be the most effective inhibitor of phosphatase synthesis (Table 4). Toluene was sometimes effective over short term

Table 4. Effect of preservatives on apparent phosphatase[a] activities in estuarine waters[b].

Treatment	Control (no preservative)	Ethanol (0.4%)	Toluene (5%) in ethanol	Sodium azide (0.01%)
24 h incubation	0.072 ± 0.005	0.076 ± 0.004	0.071 ± 0.000	0.067 ± 0.003
48 h incubation	0.739 ± 0.345	0.893 ± 0.494	0.524 ± 0.162	0.173 ± 0.008

[a] Given as absorbance at 410 nm ± standard deviation.
[b] Station 5, 5 replicates, pH 7.5.

incubations (ca. 48 h), but this was not consistent. Sodium azide however, alone or in combination, proved effective up to 120 h (the limit of testing) (Fig. 6).

Preservatives have not often been utilized, mainly because of the short incubation times generally employed (see Table 1). Berman (1970) did add 1.25% chloroform to natural water samples, but this concentration caused lysis, and both internal and external phosphatases were measured. Chloroform did not alter the enzyme activity itself. Jones (1972) also used chloroform to disrupt the cell membranes and inhibit bacterial growth. They found it to be more effective than toluene for bacteriostasis. Tabatabi & Bremner (1969) used toluene for assaying phosphatases in soils.

It was decided to use sodium azide in the current study firstly, because it was found to be an effective inhibitor of phosphatase synthesis, and secondly, it did not result in cell disruption. In this way only total extracellular phosphatase activity was measured.

(c) Filtering. To assess the differences in activities of soluble and available (soluble plus cell surface) phosphatases, water samples were filtered through 0.45 μm Millipore® filters. Filtered and nonfiltered samples were then assayed according to the standard procedure. Activities of soluble and available phosphatases were determined for 7 sampling sites, for neutral and alkaline phosphatases, and over a 96 h time course. The results for neutral phosphatase activity in surface water samples are shown in Fig. 7. Soluble phosphatase made up a mean of 24.5% of the total available phosphatase; the variation was between 9.3% and 45.1%. This data is consistent with Berman's report (1970) that the average activity of soluble phosphatase in Lake Kinneret water was 16% of the total; the range was 0 to 57%.

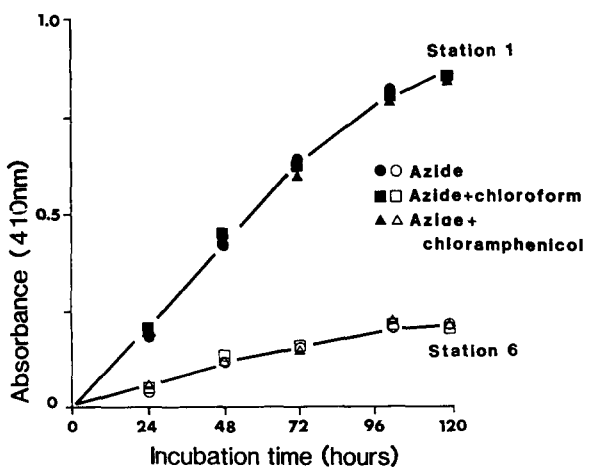

Fig. 6. Effect on apparent phosphatase activities (absorbance at 410 nm) of adding chloramphenicol and chloroform to sodium azide as preservatives.

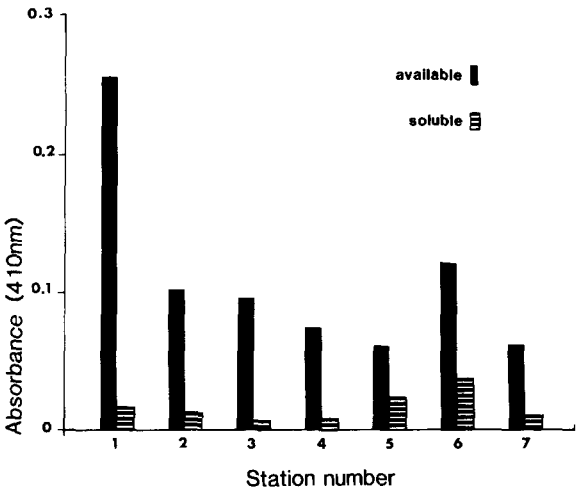

Fig. 7. Differences between the soluble and available phosphatase activities (absorbance at 410 nm) in surface waters from the seven regular sampling stations.

The relationship between bound (total minus soluble) phosphatase and chlorophyll is depicted in Fig. 8. This suggests that phytoplankton may be responsible for a large fraction of the phosphatase activity in the estuarine waters examined. However, a significant bacterial mass is likely to be associated with the phytoplankton and will also contribute to the total phosphatase activity. Jones (1972) attempted to differentiate between algal and bacterial phosphatase in lake water. Three size fractions were examined: $\leqslant 0.22$ μm, 0.22 μm $< X < 0.8$ μm and $\geqslant 0.8$ μm. The mean activity of the 'bacterial' (0.22 μm $< X < 0.8$ μm) fraction was almost 500 times greater on a dry weight basis than the 'algal' ($\geqslant 0.8$ μm) fraction. However, algal biomass was much larger than the bacterial biomass.

Many factors have been shown to be involved in the overall activities of soluble and cellular phosphatases of individual organisms. Healey (1973) characterized both forms in *Anabaena variabilis*. Cellular phosphatase was pH and temperature dependent, had a calcium but not a magnesium re-

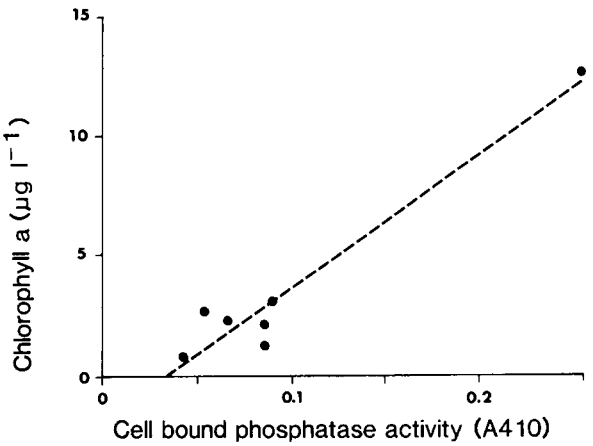

Fig. 8. The relationship between chlorophyll *a* concentrations and cell bound phosphatase activities (absorbance at 410 nm).

quirement, and synthesis was apparently stimulated by low phosphate concentrations. Lin (1977) measured intracellular, extracellular, and cell wall bound phosphatases of *Cladophora* and showed that maximum secretion of phosphatase occurred at pH 7.0. Alkaline phosphatase secretion in *Ochromonas* is stimulated by low phosphorus conditions, but acid phosphatase secretion is linear with time and apparently independent of phosphorus status (Aaronson & Patni, 1976). Lien & Knutsen (1973) located acid phosphatase near the cell surface of *Chlamydomonas* and also noted its secretion during sporulation.

General discussion and conclusions

Of the assay conditions studied here, viz., pH, magnesium requirement, incubation time and temperature, the most significant is probably pH. In an estuarine situation, where the water chemistry fluctuates markedly, it is also one of the most difficult parameters to standardize. Magnesium, though required to be added in phosphatase assays of pure culture systems, is likely to be in high enough concentrations in natural waters to obviate this.

The pH necessary to maximize phosphatase activities in assays of natural waters depends on the predominant organism(s). It is generally not feasible to determine all the organisms present or to know the pH profiles of the phosphatases. Most often, pH 8.6 has been used for assays. However, Taft *et al.* (1977) used a pH within 0.25 units of the ambient pH of the water at the time of sampling. Thus, the phosphatase activities determined in their assays were more closely related to the actual activity in the system at the time of sampling. In the current study, pH 7.5 was chosen because the phosphatase activity was often significantly higher in this region than at pH 8.6. Furthermore, the pH of this estuary varies over a wide range (7.1–8.6). By using pH 7.5, we attempted to measure potential phosphatase activity.

Sample storage prior to assay and sample preservation during assay were examined. In this study, the biomass in the water samples examined was generally quite low and storage time and temperatures used did not significantly change phosphatase activities. However, if biomass is high, secretion of phosphatases by living organisms in an enclosed system (sample container) or release of intracellular phosphatases by dying organisms could result in overestimation of extracellular phosphatase activities. Therefore it is recommended that samples be assayed as soon as possible after collection.

The requirement for the addition of a preservative to prevent microbial growth and consequently, their secretion of phosphatases, depends on the incubation time used in the assay. There are two solutions to the problem of long incubation times for samples with low phosphatase activities. One is to use the more sensitive assay with o-methylfluorescein phosphate as the substrate and incubate for a shorter time. The second is to use an inhibitor of microbial growth. Chloroform has been employed (Berman, 1970; Jones, 1972) but this causes lysis of the cells and hence total, rather than extracellular phosphatase is measured. In this study, sodium azide was found to be a more suitable preservative since it did not cause cell lysis or inhibit the phosphatases, but did prevent any observable microbial growth and consequent increases in phosphatase activity.

Whether or not samples should be filtered depends on the form of phosphatase to be examined. Since the objective of this study was to measure available phosphatase activity in the water, cell-bound as well as free phosphatases were important. In fact, the former contributed the major portion of the phosphatase activity.

It is suggested that, in order to make comparisons between the phosphatase results from different laboratories, the effects of the assay conditions examined in the present study need to be considered.

Summary

The effects of assay procedures on apparent phosphatase activities in estuarine water were determined to be as follows.

1. Added magnesium was not found to be required in the present study.
2. pH optima varied with respect to sampling site and sampling date.
3. Sample storage times of up to 72 h and temperatures of up to 22 °C did not affect the phosphatase activities.
4. The rate of cleavage of *p*NPP was linear with time to 120 hours.
5. Sodium azide, at 5 mM was found to be an adequate preservative.

The implications of these results in relation to assay procedures and data interpretation are discussed.

Acknowledgements

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