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Phosphatase activity of benthic marine algae. An overview

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Abstract

This review provides an account of the phosphatase activities of benthic marine algae and is based on reports for more than a hundred species, including cyanobacteria, red, brown and green algae. Particular emphasis is given to the use of phosphomonoesterase activity as a rapid means of assessing the phosphorus status of the alga and thus indirectly that of the environment. An understanding of the influence of environmental factors and the growth pattern of the particular alga is important in carrying out assays. For instance, the response to light differs markedly between species, especially in short-term assays, when the effect can be obvious or none. Considerations about the methodology for measuring ″alkaline phosphatase activity″ are discussed, particularly whether to simulate field conditions or to use optimum conditions. Recommendations are suggested concerning the best methodology for routine use, followed by a discussion of the future prospects for the method.

Abbreviations: APA – alkaline phosphatase activity, APase – alkaline phosphatase, DOP – dissolved organic phosphorus, G6P – glucose-6-phosphate, Ks – apparent half-saturation constant for enzymatic activity, MU – 4-methylumbelliferone, MUP – 4-methylumbelliferyl phosphate, PME – phosphomonoesters, PMEase – phosphomonoesterase, pNP – *p*-nitrophenol, pNPP – *p*-nitrophenyl phosphate, SA – surface area, SRP – soluble reactive phosphorus, V – volume

Introduction

Measurement of the phosphatase activity of whole organisms or parts of organisms is becoming a practical biochemical tool in limnological and oceanographic studies. Together with other indicators, it can help to assess the degree of P limitation in an aquatic ecosystems or a single species. The phosphatase activity also provides information about the utilization of DOP as an additional source of phosphate by bacteria and primary producers. Studies on the APA of marine organisms started with phytoplankton (Kuenzler 1965; Perry 1972), followed a few years later by an account for some smaller species of multicellular algae (Walter and Fries 1976). Although this came a decade after work on multicellular freshwater algae (Fitzgerald and Nelson 1966), there is now probably as much known about APA in marine macroalgae as in other aquatic phototrophs.

The aim of this paper is to provide an overview rather than a comprehensive cover. It includes not only the larger algae, but also benthic cyanobacteria and some comparative observations on seagrasses.

Alkaline phosphatase activity

A non-specific PMEase with optimum activity at alkaline pH and known widely as alkaline phosphatase (EC 3.1.3.1) has been reported at the external part of alga (Flynn et al. 1986; Hernández et al. 1994a), with mention of the cell wall, external membrane or periplasmic space. Since the first report of extracellular APase by multicellular marine algae (Walter and Fries 1976), APA has been measured in many other species in the red algae (Lapointe 1997; Hernández et al. 1999), brown algae (Lapointe et al. 1992; Hernández et al. 1997) and green algae (Lapointe et al. 1994; Hernández and Whitton 1996), with intertidal and subtidal examples in each group. In addition APA has been measured in benthic cyanobacteria (Yelloly and Whitton 1996) and seagrasses (Hernández et al. 1994b; Touchette and Burkholder 2000), while Hoppe (in press) has reviewed the literature on plankton.

Enzymatic assays have been done using *in vivo* (whole thallus, under field or laboratory conditions) or *in vitro* (following partial purification of the enzyme) approaches. Although APA has been detected in more than 100 benthic marine phototrophs, there are a few populations where APA could not be detected (subtidal *Halarachnion ligulatum* and *Laminaria digitata*: I.H., unpublished data) and several species where APA seems to be low (Hernández et al. 1994a, 1999) and shows no correlation with intracellular P concentration (*Ulva lactuca*: Lee (2000)).

Although nitrogen appears to the nutrient limiting productivity in much of the open sea (Falkowski and Raven 1997), records for the coastal zone often indicate that phosphorus is limiting here, at least for certain species and/or seasons (Hurd and Dring 1990; Littler et al. 1991; Lapointe et al. 1992). Evidence for this is the occurrence of a high N: P ratio in tissues of intertidal macroalgae as compared to phytoplankton (Atkinson and Smith 1983; Duarte 1992). The ratio often exceeds 30:1 (Lapointe et al. 1992) suggesting marked P-limitation (Atkinson and Smith 1983; Duarte 1992). In these cases APase probably plays an important role in the phosphorus supply during phosphate depletion. Consequently, an inverse relationship between APA and external or internal phosphate has been demonstrated, both for intertidal algae (Hernández et al. 1993, 1995) and phytoplankton (Davies and Smith 1988). Thus high phosphatase activity has been considered as an indication of phosphorus limitation of productivity (e.g. Weich and Granéli (1989) and Lapointe (1989)). PME constitutes a variable fraction of the DOP, with values for aquatic environments ranging from negligible to almost 70% (Kobori and Taga 1979; Shan et al. 1994; Karl and Yanagi 1997; Hernández et al. 2000). The DOP pool itself is sometimes greater than the SRP (Hernández et al. 1993; Thingstad and Rassoulzadegan 1995). Short-term pulses of DOP are probably common in aquatic environments, as reported from the upper intertidal following storm events which deposit algal masses which subsequently become degraded (Yelloly and Whitton 1996).

Influence of environmental variables

Light

It might be expected that the activity of an enzyme occurring at the cell surface would not be closely linked to photon irradiance, at least in the short-term, and many studies have been reported where light conditions have apparently not been closely controlled. However, more critical studies suggest clear differences between species. Some macroalgae, such as *Porphyra umbilicalis* (Hernández et al. 1992) and *Gelidium latifolium* (Hernández et al. 1995) and the cyanobacterium *Rivularia atra* (Yelloly and Whitton 1996) show no short-term dependence of APA on photon irradiance. In *Ulva lactuca*, however, APA was higher in the dark (Weich and Granéli 1989). Suppression in the dark was independent of the external phosphate concentration. Similarly APA in *Gelidium sesquipedale* and the seagrass *Zostera noltii* increased with photon irradiance (Hernández et al. 1994b, 1995). In *Z. noltii*, APA showed a saturation curve with maximum activity at a flux value greater than 130 μmol photon m^{-2} s^{-1} and this pattern was similar that of the influence of irradiance on direct phosphate uptake. This dependence of APA on light suggests a dependence on metabolic energy from phosphorylation. However, more critical studies (e.g. use of uncouplers/inhibitors) are needed to establish the underlying mechanisms.

In contrast, *Corallina elongata* achieved maximum APA in the dark, with minimum activity at relatively low irradiance (Hernández et al. 1996a). Thus APA in this species may balance the P requirements when the direct uptake of phosphate is reduced under conditions of limiting reducing power. However the dependence of APA on darkness may in some cases be indirect, resulting from the fact that APA is in-

versely related to internal phosphate (see below). A long pre-incubation in darkness may stimulate the enzyme synthesis due to a decrease in phosphate uptake, which is an energy-dependent reaction (Fogg 1973). This may apply in the studies on *G. latifolium* (see above), where a long preincubation period in the dark caused higher APA in darkness compared with an assay at 100 μmol photon m^{-2} s^{-1}, in contrast to the short-term studies showing no effect of light. Both *P. umbilicalis* and *G. latifolium* are exposed in their natural environment to a broad range of photon irradiances, which could be itself a selection factor for the enzyme to be independent of short-term changes in irradiance. Overall, the response to light seems to be species-specific. Temporal variations in APA may be a factor in the co-occurrence of species in a phosphate-depleted environment, with the PME fraction made available to different species at different times of the day (Klotz 1985). The light quality has also been shown to influence APA in several freshwater phytoplanktonic species (Wynne and Rhee 1988), with higher activity under blue light; this was not related to P limitation. Similar studies are needed on marine algae, including examples of species showing different responses to light and dark.

Temperature

The effects of temperature on enzyme activity are complex (Dixon and Webb 1979) and include the effect on stability of the APase, the effect on the actual velocity of breakdown of the complex and the effect on the enzyme-substrate affinity. In the benthic algae tested, the optimum temperature for APA is usually higher than temperatures likely to occur in nature, often with values ranging from 25 to > 30 °C (e.g. Hernández et al. (1996a)). This has a clear implication for the so-called optimum conditions for the enzymatic assay. Values of Q_{10} (ratio of the rate at a given temperature t and the rate at $t + 10$) are generally lower than 2 (i.e. activity increases less than twice over a 10 °C increase in temperature), as occurs typically in many enzymes from intertidal species (Price and Stevens 1982).

Although a rise in assay temperature for material sampled at a particular time probably leads in most cases to an increase in APA, this does not mean that activity for a particular species is necessarily highest at the time of year when field temperature are highest. For instance, APA of *Fucus spiralis* in the upper littoral zone of a Scottish shore was highest in April (when mean tidepool temperature during low tide = 14 °C), with activity values four times more than in August (mean tidepool temperature during low tide = 20 °C). In fact, APA during the winter was always higher than during the summer, in spite of assays being conducted at field temperatures (Hernández et al. 1997).

pH

Phosphatase activity of benthic macroalgae usually shows an optimum alkaline pH for activity, mostly between 8.7–9 (Hernández et al. 1996a), a value likely in the field only in shallow pools of the upper littoral zone, where high pH values are often reached (Larsson et al. 1997). In that case, as may occur for *Fucus spiralis* (Hernández et al. 1997), there is presumably an especially high phosphate supply from PME during periods with high rates of carbon fixation, such as when low tides coincide with saturating irradiance for photosynthesis. Assays of APA of intertidal macroalgae at typical sea water pH (*c.* 8.3) indicate values for activity of about 70–80% for those at the optimum pH.

A complication in assessing the likely effect of pH in nature is the fact that the optimum pH is known for some other organisms to depend on the substrate concentration used in the assays (mammalian tissue, Fedde and Whyte (1990); the freshwater diatom *Synedra acus*, Hantke and Melzer (1993); several freshwater strains of *Calothrix*, BAW, unpublished data). In all these cases an increase in substrate concentration led to a shift of the pH optimum towards the alkaline range. However, the majority of assays on intertidal seaweeds have been conducted at saturated substrate concentrations, so it is important to establish whether there is an influence of substrate concentration on the optimum pH for APA in these organisms.

Salinity

The APA of benthic intertidal macroalgae is affected by salinity (Hernández et al. 1995; Lee et al. 1999). Enzymatic activity has been shown to be very low under low salinities and to increase with increasing salinity up to 45–50‰, where maximum APA was found (Hernández et al. 1995). However, several days of exposure under low salinity caused an enhancement of APA in *Gracilaria tenuistipitata* by the hyposaline stress and a decrease in tissue P (Lee et al.

1999). The effect of salinity includes both an osmotic component and one which increases with concentration of particular ions. The two effects can be distinguished if some of the main salts contributing to changes in salinity are replaced with a non-electrolyte, such as mannitol (Hernández et al. 1995). The effect of salinity on APA seems to be attributable not only to the ionic strength, but to a specific effect of particular cations as Na^+ or Mg^{2+}. As the intertidal zone may be subject to a broad range of salinity conditions during various combinations of the tidal cycle and climatic conditions, ranging from almost fresh water after heavy rain to high salinity at high irradiance and strong desiccation, the effect of these different salinity conditions on APA requires further investigation.

It is unclear whether the response to increased salinity is merely an enhancement of APA under stressful conditions, or has adaptational significance in enhancing P availability. The latter might apply, for instance, because of increased difficulty in taking up phosphate or greater availability of DOP (Lee et al. 1999). If it is of adaptational significance, it seems probable that a variety of responses to salinity will be found, just as there is a variety of responses to light. At least another quite difference response is known. A laboratory culture of *Calothrix viguieri*, originally isolated from a mangrove root, showed very low APA in saline medium, but a marked increase within a day of transfer to freshwater medium; the change was associated with the development of multicellular hairs where the APA is located (Mahasneh et al. 1990).

Phosphate

Many studies have revealed that APA of macroalgae usually increases markedly during the cultivation in a phosphate-deficient medium and is decreased at high external phosphate concentration (Weich and Granéli 1989; Hernández et al. 1995). Orthophosphate is a potent competitive inhibitor of APase (McComb et al. 1979) and it is therefore to be expected that APA will be suppressed in the presence of high phosphate concentration. However, during cultivation of many benthic macroalgae at a constant high (> 300 $\mu g\ L^{-1}$ P) phosphate concentration during several days, APA is not suppressed totally (e.g. *Porphyra umbilicalis*: Hernández et al. (1992); *Gelidium sesquipedale*: Hernández et al. (1995)). In addition, macroalgae from a hypereutrophic location in the Florida Keys show high APA values both in winter and summer (Lapointe et al. 1994). This evidence suggests the existence of two different phosphatase enzymes with significant activity at alkaline pH: one adaptive, whose rate of synthesis is regulated by phosphate concentration, and one constitutive, independent of the external phosphate levels (Kuenzler 1965; Siuda 1984), as has been reported for *Chlamydomonas* (Boavida and Heath 1986).

Beside the existence of different phosphatase enzymes, there are other possible explanations for the absence of a total repression of APA by external phosphate in marine macrophytes. When the effect of phosphate is tested, the substrate/phosphate ratio during the assays is usually high, as the activity is generally measured under substrate saturation. These conditions assure that little or no inhibition may be caused by phosphate (McComb et al. 1979). The study of the inhibitory effect of phosphate on APA in future studies should therefore measure the enzymatic activity under low substrate concentration (much lower than saturation) and high phosphate concentration. For instance, a study in a mesotrophic lake (Hernández et al. 1996b) showed that phytoplanktonic APA assayed with 100 nM ^{32}P-G6P was inhibited significantly by 1 μM phosphate without any period of pre-incubation.

Apart from the substrate:phosphate ratio effect, which may affect any conclusions about the phosphate inhibition of APA, the enzymatic activity of some benthic macrophytes is poorly related to external phosphate concentration (*Corallina elongata* Hernández et al. (1996a), the seagrass *Zostera noltii*; Hernández et al. (1994b)). Similarly, some planktonic algae produce phosphatases that are not easily inhibited by phosphate addition (Stewart and Wetzel 1982; Pick 1987), and may also be the case for certain benthic marine macrophytes (*Ulva lactuca*: Lee (2000)).

A constant high external phosphate concentration will eventually cause a high tissue P and therefore a significant inverse correlation between APA and tissue P may be expected. Some cell phosphate pools have been shown to influence APA as surplus phosphorus, polyphosphates or total phosphorus (Jansson et al. 1988). In benthic macroalgae, an inverse correlation between APA and cellular particulate phosphorus or total phosphorus has been found in *Porphyra umbilicalis*, a red alga with a structurally simple thallus (Hernández et al. 1993). Similarly, Weich and Granéli (1989) suggested a negative correlation between APA and the P status in meristematic areas of

Ulva lactuca. In contrast, structurally complex macroalgae such as *Fucus spiralis* can show different responses without an immediate interpretation of the relationship (Hernández et al. 1997).

Based on the inverse relationship between APA and different phosphorus fractions, different authors have suggested that high APA values can be used as an indicator of P-limitation (Fitzgerald and Nelson 1966; Lapointe 1989; Hernández et al. 1993), though Cembella et al. (1984) raised many problems in its use for phytoplankton. Among the marine algal examples, the high APA value at the end of the gametophytic cycle of *Porphyra umbilicalis* (Hernández et al. 1992) occurs when growth seems to be P-limited (Flores-Moya et al. 1997). The low APA values in *Caulerpa taxifolia* provided evidence (with other indicators such as tissue N and P levels) that productivity was unlikely to be severely limited by nutrients (Delgado et al. 1996). Also Pérez and Romero (1993) showed that APA in the seagrass *Posidonia oceanica* was repressed by increasing levels of nutrient availability. APA in *Fucus spiralis* from the S-E. coast of Scotland was very low throughout most of the year, when N seems to limit growth. The only period suggesting P limitation was coincident with a marked increase in APA (Hernández et al. 1997). Caution is, however, needed when interpreting results. For instance, some macroalgae shows very high APA values which are related to high surface area volume ratio (SA:V) rather than cellular P (see below).

Other aspects of the biogeochemical environment

Other aspects of the biogeochemical environment can also influence APA. Macroalgae from temperate siliciclastic environments (influenced by terrigenous organic matter and with low N: P ratios: Nixon et al. (1980)) have significantly lower APA than macroalgae from tropical carbonate-rich environments (Lapointe et al. 1992), which generally have low phosphate concentration (Hines and Lyons 1982). Macroalgal tissue from the siliciclastic sites also have a significant higher P content than those from a carbonate-rich environment (Lapointe et al. 1992).

Biological considerations

APA is also influenced by other biological factors, including interspecific differences, type of tissue and the SA:V ratio. Some species shows undetectable or very low maximum APA. For instance, APA values below the detection limit have been found in the subtidal *Halarachnion ligulatum* and *Laminaria digitata* and the estuarine *Ulva rigida* (Hernández et al. 1999). Other macroalgae, as *Bryopsis pennata* (Lapointe et al. 1992), *Laurencia intricata* (Lapointe et al. 1994) and *Chondrus crispus* (Hernández et al. 1999) may show very low enzymatic activity (less than 1 μmol g dry wt^{-1} h^{-1}). APA seems to be quite low in some species of the genera *Ulva* and *Enteromorpha*, both from estuarine and intertidal ecosystems (Hernández et al. 1994a, 1999; Lee 2000). These species probably satisfy most of their phosphorus demand directly from inorganic phosphate so that they do not compete for PME with other species. It might also occur that APA is repressed due to high cell quotas for phosphorus when the assays were performed. Enzymatic activity does not seem to be influenced by cutting the thallus to produce tissue segments (I.H., unpublished data). Therefore, it is possible to restrict assays to large parts of thallus, though it is essential to consider intra-thallus variability. For instance the tips of *Fucus spiralis* (meristematic, younger parts) were found to exhibit the highest APA (Hernández et al. 1997). The activity decreased towards a constant value in the middle and basal parts of the thallus. Total cellular P and N showed the same trend, whereas tissue C increased from the apex to the basal part of the thallus. Therefore a profile of the thallus of *F. spiralis* showed a significant positive correlation between APA and tissue P and N. In *Zostera noltii* the highest activity was found in the leaves, with minor contributions in the stem and the underground parts of the plant (Hernández et al. 1994b).

The SA:V ratio also has an important influence on APA, due to the enzyme being located at the outer part of the thallus (Hernández et al. 1994a). The influence of SA:V ratio on APA was tested in several intertidal benthic macrophytes collected at Tyne Sands (S.E. Scotland) and El Chato (Cádiz, Spain) (Hernández et al. 1999). Pooling all these species, it was found that APA was not related to tissue P (Figure 1) or N:P ratio (Hernández et al. 1999). However, when plotting APA versus SA:V (both on logarithmic scale), a significant positive relationship was evident (Figure 2). Plants with low SA:V ratio tended to have lower APA than plants with high SA:V ratio. The regression equation showed that SA:V accounted for 29% of the variance in APA. The relationship between APA and SA:V improves if the three "anomalous" species with high SA:V but low APA (*Enteromorpha intestinalis*, *Ulva rigida* and *Porphyra* sp.)

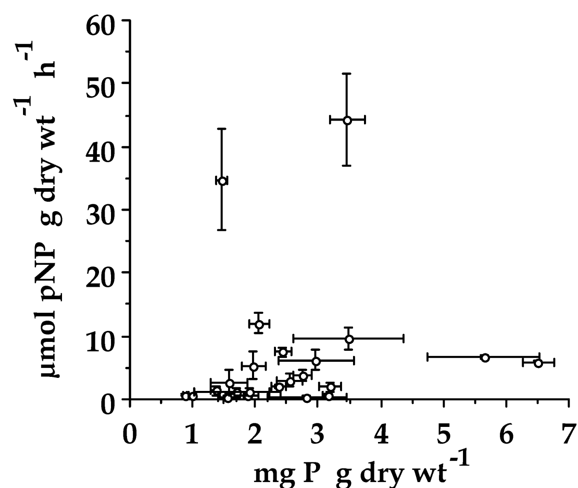

Figure 1. Alkaline phosphatase activity of benthic marine macrophytes from two intertidal systems as a function of their total tissue P. Values represent means (n = 3 − 16) ± SD. Modified from Hernández et al. (1999)

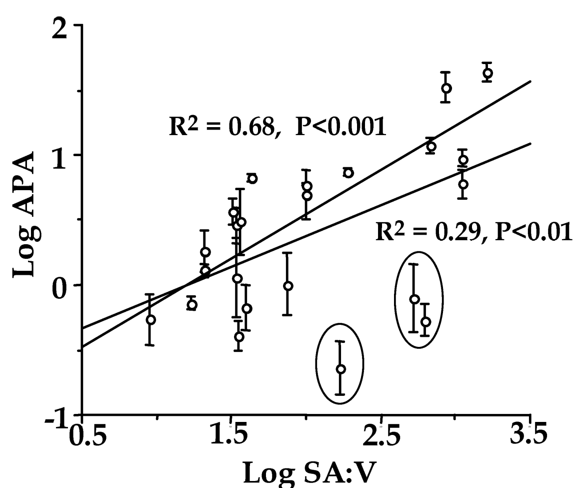

Figure 2. Regression analysis of alkaline phosphatase activity with relative surface area (SA:V) in different benthic marine macrophytes from two intertidal systems. Values of APA are mean (n = 3 − 16) ± SD. Note that the two axes are logarithmic. The best line fitted represents the regression analysis without the three points enclosed by ovals. Modified from Hernández et al. (1999).

are not taken into account (see Hernández et al. (1999) for further discussion). In that case 68% of the variance in APA was attributable to SA:V.

Therefore differences of SA:V among marine macrophytes should be considered when comparing phosphatase activities between different species. However the relation between APA and SA:V must be treated with caution. Substantial remaining variation can be assigned to species-specific variation in APA, as maximum enzymatic activity may vary considerably (about 10-fold) within a single species, depending on several biological and physicochemical factors such as total cellular P. In any case the strong relationship between APA and SA:V within a narrow size-range supports the existence of a coupling between physiological and morphological properties in algae. For instance, APA in cyanobacteria (Whitton et al. 1990, 1991) may reach values much higher than in macroalgae. The importance of the SA:V ratio has been pointed out in other physiological processes, as shown by nutrient uptake rates (Hein et al. 1995) or growth rates across photosynthetic organisms (Nielsen et al. 1996).

Methodological considerations

Introduction

It seems probable that the majority of benthic marine macrophytes have potential ability to hydrolyze PME under natural conditions. However, the ecological significance of this ability to determine P availability relies on the existence of adequate DOP, a high affinity for these substrates and a significant effect of the P supply from PME on macroalgal grow (Cembella et al. 1984). These conditions are not always easy to demonstrate and some have clear methodological implications.

Most studies on APA in phototrophs have used indirect methods to estimate the enzymic activity. Such methods involve addition of artificial substrates whose non-phosphate moiety is coloured or fluorescent. Ecologists have then tried to relate the enzymic activity shown by the assays to conditions found in nature (Lapointe et al. 1992; Hernández et al. 1995). However, it is not always easy to interpret the ecological significance of some past studies, particularly when assays have been conducted entirely in the laboratory.

Assays in the field or in laboratory?

In order to measure APA, plants are usually collected in the field and transported to the laboratory. Then, the plants are maintained under laboratory conditions before the assays. These two operations usually imply that, at best, assays are run several hours after removal from field. In any case APA measurements should begin as soon as possible after collection.

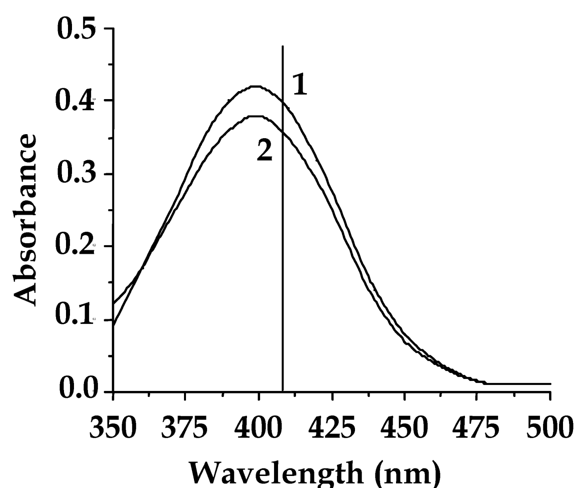

Figure 3. Absorbance spectra of paranitrophenol (pNP) resulting from the measurement of alkaline phosphatase activity in a benthic macroalgae. Spectrum 1: before freezing a sample of assay medium in liquid nitrogen. Spectrum 2: after thawing sample of assay medium. Vertical line indicates 410 nm.

Long (overnight) pre-incubations under laboratory conditions before the assays may bear dubious relation to the physiological-nutritional state of the macrophyte at the time of collection. Similar considerations have been proposed for photosynthesis measurements (Henley 1993).

These problems can be avoided when APA assays are performed directly in the field. If pNPP is used as a substrate for the enzymatic reaction, a sample of the assay medium (after a phosphatase reaction run for 1 h) can be placed in a capped vial and immersed in liquid nitrogen. Then vials can be quickly defrosted in the laboratory and the absorbance read in a spectrophotometer. This method has been successfully employed to measure APA of macroalgae (Hernández et al. 1993, 1997). The freezing-thawing changes affect just slightly the absorbance spectrum of pNP or the readings at 410 nm, which is the wavelength generally used to infer the enzymatic activity (Figure 3). If time of thawing and reading is carefully standardized, this procedure may also be valid when a fluorigenic substrate is used (IH, unpublished data).

Choice of the substrate

Studies in marine macroalgae generally employ the chromogenic, artificial substrate pNPP to measure APA. Lately, fluorogenic substrates, as MUP have been used in benthic cyanobacteria and macroalgae (Hernández and Whitton 1996; Yelloly and Whitton 1996). Fluorogenic substrates show higher sensitivity than pNPP (Pettersson and Jansson 1978), but do not give them greater advantage in studies about macroalgal APA, as the biomass used in the assays (and therefore the amount of enzyme) is usually high enough to render sufficient pNP, the coloured product released from pNPP, during a short (< 1 h) time of incubation. On the other hand, the affinity of algal APase for fluorogenic substrates is usually higher than for pNPP (Whitton et al. 1998). However the simplicity of the colorimetric method makes pNPP the preferred substrate to measure APA in benthic macroalgae, especially if enzymatic activity is measured at maximum velocity.

Both chromogenic and fluorogenic substrates have the limitation of being artificial and therefore they are probably not representative of natural substrates (which are largely unknown). Therefore their usefulness is probably limited to the determination of qualitative and not quantitative relationships between APA and the environmental variables (Siuda 1984). Studies in lake plankton have used radiometric methods which permit the use of ″natural″ substrates. Bentzen et al. (1992). used ^{32}P-ATP to represent the available fraction of the PME pool, concluding that PME contributed significantly to total P uptake, especially in bacteria, in a system of low phosphorus availability. Hernández et al. (1996b) showed that the substrate ^{32}P-G6P provided a sensitive way to assess PMEase activity and the fate of the PME pool in a P-limited lake. It was suggested that the rate of phosphoryl uptake from PME represented a high percentage (> 40%) of the algal phosphate demand in summer. Studies using radiolabelled natural PME should be included in future studies on APA of macroalgae.

Substrate concentration: field conditions or potential maximum activity?

Another important conflicting aspect concerns the ″optimum″ conditions for measuring APA. Should APA be assayed at maximum velocity or at substrate concentrations close to those existing in the field? What does ″optimum″ really mean? The answers depend on the aim and the approach to the problem. Biochemists usually assay enzymatic reactions when the enzyme is extracted and purified from the organism and at standard and controlled conditions of alkaline pH and temperature. These conditions are less satisfactory for ecological studies as activities measured in this way do not reflect conditions in nature.

However ecologists also disagree about the most appropriate conditions for APA assays. Standard conditions (e. g. saturating substrate concentration, 20–25 °C, pH 8–8.3) are useful if the APA of different species are compared (e.g. Delgado and Lapointe (1994) and Hernández et al. (1994a)). However, these conditions are probably not the most suitable if it is desired to measure APA under conditions *in situ*.

The use of substrate concentration at saturation level should give information on the potential maximum activity. McComb et al. (1979) recommend that the substrate concentration that supports maximum activity can be estimated roughly as 20 times the Km. However, many intertidal algae exhibit apparent negative cooperativity for the hydrolysis of pNPP (Hernández et al. 1995, 1996a). This property, defined as an apparent inhibitory action of one ligand on the binding to, or reaction of another with the enzyme, has been described for purified APase (Levitzki and Koshland 1976). In these cases two apparent Ks values for pNPP can be deduced. The higher affinity value is usually less than 50 μM, therefore pNPP concentrations ca. 800 μM guarantee maximum velocity of the ″high affinity″ phase. However, the lower affinity value is about 2 mM, which means that a concentration of 40 mM pNPP would be needed to reach maximum velocity! Such a concentration is totally unrealistic, more than twice the values recommended by McComb et al. (1979) for any phosphatase assay (16 mM), so in algae showing an apparent negative cooperativity we suggest that the lower Ks value should be used to calculate the concentration at saturation level.

On the other hand, in order to minimize the effects of product inhibition or substrate depletion, the initial concentration should be sufficiently high to ensure that no more than 10% is hydrolyzed during assay (McComb et al. 1979). In a standard assay (1 h) with *Fucus spiralis* this condition was met when pNPP concentration was > 250 μM, whereas lower concentrations of MUP (ca. 5 μM) fulfilled this condition (Hernández and Whitton 1996). The high sensitivity of the fluorogenic substrate MUP enables assays of APA at 1 μM (Yelloly and Whitton 1996). Therefore pNPP seems to be an excellent (although artificial) substrate to be used when working at potential maximum activity, whereas MUP seems more appropriate if APA is assayed at more realistic PME concentrations in nature.

Estimation of APA at natural PME concentrations implies the need for a reliable method to measure PME in the water. There has been much effort in this chemical analysis, due to the diversity and complexity of the compounds present in the water. PME concentration can be determined as the difference between SRP measurements before and after exhaustive hydrolysis with calf intestinal mucosa APase (Francko and Heath 1979; Hernández et al. 1993; Huang and Hong 1999). If the PME concentration is such that measurements would be below the detection limit (< 60 nM) of the standard method of Murphy and Riley (1962) for SRP, as may occur in oligotrophic fresh waters, the sample can be previously concentrated using a ion-exchange chromatographic column (Hernández et al. 2000). However, the latter is impossible using sea water, as the high salt content interferes with anion retention on the column (Camarero 1994). An improved method of measuring PME in natural waters was proposed by Shan et al. (1994). This involves the estimation of the concentration of PME by enzymatic flow injection, using an immobilized *Escherichia coli* APase packed-bed reactor. Concentrations of PME in rivers are usually in the low micromolar or nanomolar range, but may represent a significant percentage (>10%) of the total dissolved phosphorus (Shan et al. 1994; Hernández et al. 2000). In coastal sea water, the PME concentration is sometimes greater than SRP (Hernández et al. 1993; Thingstad and Rassoulzadegan 1995). The bioavailable DOP pool, however, represents a small fraction of the bulk DOP within coastal environments (Benitez-Nelson and Buesseler 1998; Suzumura et al. 1998).

Karl and Yanagi (1997) developed a new method (by a combination of two independent methods for P determination) to estimate the UV-labile phosphorus fraction of the total dissolved P pool ($P_{UV\text{-}L}$, which contain primarily monophosphate esters) in oceanic waters. According to this analytic method, the $P_{UV\text{-}L}$ in the upper portion of the water column of the North Pacific Ocean constituted, on average, more than 50% of the total dissolved P pool, with concentrations ranging from 100 to 200 nM P. More recently, Kolowith et al. (2001) using tangential-flow ultrafiltration and solid-state ^{31}P nuclear magnetic resonance spectroscopy charactherised the dominant compound classes of marine high-molecular weight (1–100 nm size fraction) DOP. Their study revealed that, in samples from different oceanic regions where concentrations were always lower than 100 nM, phosphate esters (PME and P diesters) represented the dominant compound class (75%) of ultrafiltered DOP.

Therefore, if we estimate the kinetic parameters (V_{max} and Ks) for the enzymic reaction and the PME concentration in the water, it is possible to calculate the cleavage rate under natural conditions (Heath 1986; Hernández et al. 2000). The assumptions have to be made, however, that the substrate used in the assays resembles naturally occurring PME and that the enzyme exhibits kinetic parameter values for all naturally occurring substrates similar to those determined for the model substrate.

Buffers

When estimating APA, algal samples are usually incubated under constant pH. The assay medium usually has a typical (34–37‰) sea water salinity and, as the chemical composition of sea water is relatively constant, the unbuffered pH usually ranges only between pH 8.0 and 8.3. The majority of pH profiles for APA in macroalgae show very little change of activity in this range (Hernández et al. 1995), as the optimum is usually at more alkaline pH. Besides, there are no significant pH changes during a typical assay (1–2 h of incubation) with either pNPP or MUP as substrate (García-Ruiz et al. (1997); IH, unpublished data). Therefore there is probably no need for buffering in the majority of routine assays for marine macrophytes. The buffer concentration used in assays is typically in the millimolar range or even higher (Walter and Fries 1976; Lapointe et al. 1992; Hernández et al. 1994a), and this makes the assay medium very different from that of a natural environment. Only if a pH profile is constructed, or if one desires to assay at optimum (showing the maximum activity) pH, which may itself depend on the substrate concentration (see 3.3), is buffering really justified in marine environments.

Temperature

Past studies have mostly been conducted at one particular temperature for a whole research programme or carried out under natural conditions *in situ*. The standard temperature has often been 20–25 °C (Burkholder and Wetzel 1990; Pérez and Romero 1993; Hernández et al. 1995), which are usually lower than the optimum (maximum) temperature for APA. However the conclusions about a seasonal study of APA are strongly influenced by the approach used. Hernández (1996) showed that correlations between APA and total cellular phosphorus in *Porphyra umbilicalis* differed if the assays were performed at standard (25 °C) or algal canopy temperature. There was no correlation between the two variables using the former approach, but a highly significant one using the latter approach. The APA profile is also totally different depending on the way of considering temperature in a seasonal study in *Fucus spiralis* (Hernández et al. 1997). Seasonal changes of RuBisCo activity in *Laminaria hyperborea* were also very different depending on the standard temperature (25 °C) or the temperature at which the kelp would have living on the shore (Küppers and Weidner 1980).

The ″optimum″ viewpoint is far from an ecological approach because it may depend on the given pH (McComb et al. 1979) and becouse optimum temperatures for APA may be ecologically irrelevant. In macroalgae maximum APA is usually found at temperatures of 30 °C or greater (e. g. Hernández et al. (1996a)). Some phytoplankton algae phototrophs show maximum APA at 40 °C or higher (Grainger et al. 1989; Rivkin and Swift 1980), well above the maximum temperature of natural waters. Then we may ask if in such cases APA assays should be performed at these temperatures.

Period of incubation

Some of the non-phosphate moiety (pNP or MU) can be released to the medium subsequent to completion of routine assays with marine macrophytes (Hernández and Whitton 1996). This leakage depends on the duration of the APA assays and may lead to severe underestimation of the enzymatic activity if assays are run for a short time. However the leakage becomes negligible in relation to the total moiety released when the time of incubation is longer. In *Fucus spiralis* the retention of pNP and MU had a negligible influence on results when assays were performed at least for 60 min (pNPP as substrate) or 30 min (MUP as substrate) (Hernández and Whitton 1996). However the results showed considerable differences between species.

Estimate of biomass

The specific APA (ratio of APA to a biomass estimator) has been standardized to different biomass estimators, including dry weight, ash-free dry weight, carbon, cell number, chlorophyll or protein. The choice of variable to measure has often been a reflection of what is most convenient for a particular or-

ganism or circumstance, but dry weight or ash-free dry weight are probably the most suitable provided that there are no problems such as difficulty in removing mineral materials. APA of *Porphyra umbilicalis* showed the highest correlation with total cellular P when dry weight was used as a biomass estimator (Hernández 1996). The APA related to total dissolved proteins or total carbon also showed a significant correlation with total P, but the correlation was very low when related to chlorophyll. The chlorophyll content of a thallus may vary markedly depending on ecophysiological conditions (Gantt 1990). Therefore the use of chlorophyll to estimate biomass should be avoided wherever possible, and other biomass estimators showing lower turnover should be used. In any case, the units of specific APA are usually selected for convenience or practical reasons, and all have limitations that should be recognized in the interpretation of the data.

All these considerations might suggest that the effective APA for marine benthic macrophytes cannot be estimated from a simple phosphatase measurement. However we think that there is no need for such pessimism: careful evaluation of the best methodology for a particular study should make it possible to approximate the *in situ* hydrolysis rate of PME. At the very least the enzymatic activity would be more realistic than a simple number obtained under standard conditions seldom met in nature.

Future prospects for use of APA in monitoring

This review suggest many areas where further research is important, but are especially relevant to marine macrophytes. The development of the use of certain species for environmental monitoring is attractive, not just because of the practical advantage, but also because it would encourage greater rigour in methodology. Suitable species would need to show a reproducible response of APA to phosphorus stress and hence indirectly to the phosphorus status of the environment. They should be widespread, easy to identify, and preferably should be finely branched (high SA:V ratio). APA should show a strong response to the internal phosphorus concentration. Genera of macroalgae whose species fulfil at least some of these conditions include *Gelidium*, *Cladophora*, *Polysiphonia* and *Stypocaulon*, but almost certainly it will prove necessary to characterize individual species.

Assays of APA have been used to measure phosphate assimilation in macroalgal species subject to different N loading rates (Thompson and Valiela 1999). Recently, a biosensor has been constructed to detect heavy metals from inhibition of APA in *Chlorella vulgaris* (Durrieu and Tran-Minh 2002). These studies open up new possibilities to use APA for environmental monitoring.

The need to increase the use of radiolabelled natural substrates has already been mentioned. Substrates such as ^{32}P-G6P or other natural PME should be encouraged in ecological studies on the significance of APA in macroalgae. Techniques such as gel filtration chromatography used to purify APase in the dinoflagellate *Prorocentrum minimum* (Dyhrman and Palenik 1997) should be applied to marine macrophytes. The purified protein has represented a unique antigen to develop an antibody-based method to detect the presence of the cell-surface APase and to examine the relationship between APase and phosphorus deficiency (Dyhrman and Palenik 1999, 2001) and may serve to examine other environmental stresses which seem to enhance APA, as emersion in intertidal algae (Hernández et al. 1997). An antibody probe has also opened the possibility of estimating APA in a single cell by combining the technique with flow cytometry. The development of molecular markers as done for phosphate and other nutrient uptake pathways in marine pico-cyanobacteria (Scanlan and Wilson 1999; Mann 2000) is an alternative approach, which might eventually permit the recognition of a range of phosphatases related to the hydrolysis of different types of phosphate ester. A useful technique using an insoluble fluorogenic substrate (enzyme-labelled fluorescence) for APase has recently been introduced to detect and quantify the enzymic activity in marine phytoplankton (González-Gil et al. 1998), combining the enzymatic assay with epifluorescence microscopy and flow cytometry. The future development in marine macrophytes offers a new level of resolution and sensitivity at the single cell level that can provide new insights into the P nutrition of these organisms.

It is also worth considering the role phosphatase activities for marine macrophytes in nature. The majority of studies so far probably deal with organisms taken from moderately eutrophic environments. The fact that they can develop high APA under P stress nevertheless suggests that enhanced P availability is likely to be important for the organisms. This applies even more to environments where phosphate levels

are likely to be very low and often mostly as organic phosphate, such as algal reef ridges or the algae growing among corals along unpolluted tropical coasts (Lapointe et al. 1994; Schaffelke 2001). Crustose reds involved in carbonate deposition there seem likely to show very high phosphatase activities. It will be important to study PDE and not just PME activity, because of the possibility that diesters may be more important in such environments, as they appear to be in some low nutrient soils (Turner and Haygarth 1999).

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